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Cover Photograph

A Y-79 human retinoblastoma cell treated with pigment epithelium-derived factor (PEDF). Photograph courtesy of Dr. Gerald Chader, Chief, Laboratory of Retinal Cell and Molecular Biology, NEI. Dr. Chader's report begins on page 255.

National Eye Institute

Annual Report
Fiscal Year 1994

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Statement of the Institute Director

Carl Kupfer, M.D.

In this first year of our new research plan *Vision Research—A National Plan: 1994-1998,* vision researchers have made significant progress in achieving the stated goals and objectives that are considered so important to improving the visual health of the American people. During this fiscal year, 1,210 research grants were funded for nearly $235 million, and an additional 19 research and development contracts were funded for approximately $7.8 million. Another $30 million was expended in support of the intramural research program. As this year's annual report demonstrates, the investment by American taxpayers in vision research has led to several important breakthroughs by our intramural and extramural laboratories and clinical scientists and has continued to improve our understanding of the pathological processes involved in diseases of the eye and disorders of vision.

As an example, the National Eye Institute (NEI)-supported intramural scientists continue to provide new and important data on the role and function of the visual motor system. A study investigating the control of movement by visual input and the systems in the brain that perform this vital function is under way. Recently, these scientists tested the hypothesis that the spread of activity in the superior colliculus, a brain structure critical to rapid eye movement, controls the amplitude of such movement. Minute injections of a neurotransmitter inhibitor into the superior colliculus changed the direction of the eye movement. This suggests that not only the end-point but also the path taken by the eye is influenced by the superior colliculus; this information is profoundly important not only for understanding how the brain controls eye movement but also how it controls movements in general. This remarkable advance in understanding the sensory-motor system creates opportunities to examine new mechanisms of controlling movement and could lead to a better understanding of how to control disease-induced disorders of eye movement control.

Intramural researchers have also continued their important studies on the role of aldose reductase (AR) in inhibiting ocular complications associated with diabetes. They have developed an animal model (galactose-fed dogs) that develops both the clinical and histological lesions associated with all stages of diabetic retinopathy. Using this model, investigators have conducted studies to image noninvasive cataract formation and to measure the levels of AR in the lens *in vivo.* With this technique, they have also demonstrated a direct correlation between inhibition of AR in the lens and the prevention of the retinal pericyte degeneration, which leads to diabetic retinopathy.

Intramural scientists are continuing their basic and clinical studies on the natural history and the role of the ornithine aminotransferase gene in the development of gyrate atrophy (GA), an inherited degenerative retinal disorder. The research is currently directed at genetically altering the defective gene and culturing skin tissues that will be used as a transfer vehicle for inserting the altered gene in GA patients. This research may lead not only to an effective therapy for GA but may be extended to genetic interventions for other blinding genetic disorders.

Scientists in NEI's intramural laboratories have discovered several genes that may be implicated in macular degeneration, a leading cause of blindness in older Americans. In addition, a new gene has been cloned that could be the defective gene in Bardet-Biedl's syndrome, a hereditary disease of blindness and mental retardation. These investigators also have discovered a protein that promotes the survival of neurons within the central nervous system. This protein (PEDF) promotes maturation of photoreceptor-like cells derived from the retina. It also greatly lengthens the life-span of brain neurons in tissue culture experiments. With these characteristics, future researchers will explore whether PEDF can be used in transplantation procedures that could be helpful in conditions such as Parkinson's disease.

NEI intramural scientists are examining the role of cell adhesion molecules and cytokines in the development of ocular inflammatory disease. Cell adhesion molecules are surface proteins that are important for antigen sensitization and leukocytes' migration to inflammation sites. Researchers are currently investigating compounds that block cell adhesion molecules as a treatment for uveitis. They have been able to inhibit significantly the development of endotoxin-induced uveitis in mice with a single injection of anti-Mac-1 antibody. Research is now under way to develop and test topically administered substances that can block critical cell adhesion molecules. If animal studies are successful, scientists plan to test therapies based on blocking critical cell adhesion molecules and cytokines in the clinical trials of uveitis patients.

Scientists in NEI's intramural program have been studying the trabecular meshwork, a tissue in the eye that has been implicated in the development of open-angle glaucoma. In a series of experiments, investigators have analyzed the proteins present in the four quadrants of the normal trabecular meshwork and are now examining the differences between normal tissue and glaucomatous trabecular meshwork and the changes that precede glaucoma. Such results would offer important clues on the causes of open-angle glaucoma and may provide the foundation for the development of effective therapies.

The most common causes of blindness in the United States are associated with the aging process. NEI epidemiologists have initiated a natural history study to assess the clinical course, prognosis and risk factors of age-related macular degeneration (AMD), and cataract. Further investigation of the risk factors in the development and progression as well as their pathophysiologies may lead to methods for preventing these debilitating ocular disorders. Additionally, the effects of pharmacologic doses of antioxidants—vitamins C and E and beta-carotene—and zinc on the incidence and progression of AMD and antioxidants on the incidence and progression of lens opacities will be assessed as part of a randomized clinical trial, using the cohort of 4,600 patients developed for the natural history study.

NEI-supported extramural scientists have reported the loss of neuronal cells in the brains and retinas of acquired immunodeficiency syndrome (AIDS) patients, which is thought to contribute to the neurologic and retinal dysfunction often associated with human immunodefiency virus (HIV)-1 infection. Retinal ganglion cells involved in the transmission of information from the retina to the brain appear to undergo physical changes in these patients. These scientists have shown that a complex web of interactions between the cells of the immune system and the neurons is involved, involving a protein known as gp120. This protein is found on the surface of the HIV virus. They have found that adding gp120 to retinal ganglion cells growing under laboratory conditions causes injury to the cells. They have also found a receptor antagonist that may give protection from gp120 neurotoxicity, which may one day lead to the development of the means to prevent the neurological manifestations of AIDS.

An abnormal proliferation and leakage of retinal blood vessels leading to loss of vision are characteristic of a number of conditions in which the oxygen supply to the retina is ob-

structed. These conditions include severe diabetic retinopathy, retinal vascular occlusions, and retinopathy of prematurity. Recent studies supported by the NEI have demonstrated that a protein called vascular endothelial growth factor (VEGF) is present in greatly increased amounts in the ocular fluid of patients with these conditions. Production of VEGF is also enhanced by experimental procedures that decrease the oxygen supply to the retina. As VEGF is well known to be capable of stimulating blood vessel growth in the eye, these new studies indicate that VEGF may play a major role in diabetic retinopathy and other ischemic eye conditions. Investigators will expand the search for ways to either block the production of VEGF or to block its ability to stimulate the growth of new blood vessels.

Because several inherited macular degenerative diseases share significant similarities to AMD, NEI-supported scientists are searching for defective genes in affected families. Investigators have localized gene mutations to specific chromosomes for three forms of inherited macular degeneration. These investigators have shown that a mutation in one of these genes causes macular degeneration, while a different mutation in the same gene causes retinitis pigmentosa (RP). The information gained from these studies should help in understanding the cause of the more prevalent AMD.

During this last year, investigators from the NEI-supported Prospective Evaluation of Radial Keratotomy (RK) reported the results of their 10-year followup study. The data continue to indicate that RK remains a reasonably safe and effective method for correcting myopia (nearsightedness). However, more than 40 percent of RK-operated eyes continue to have a gradual shift toward farsightedness. This finding suggests that some people who have RK may need glasses at an earlier age for poor close-up vision, a common problem after age 40, than if they had chosen not to have the surgery. Data on quality of life, symptoms related to glare and fluctuating vision, and subjective visual function remain to be analyzed.

Last year NEI-supported researchers reported the localization of the gene for juvenile onset glaucoma, a form of the disease characterized by early adulthood onset and elevated intraocular pressure, to chromosome 1. These researchers have now mapped the gene to a region of chromosome 1 extensive enough to contain approximately 20 genes. Once the exact location of this gene is determined, the gene can be cloned and sequenced. The hope is that the deoxyribonucleic acid (DNA) sequence of the juvenile glaucoma gene will reveal clues about the fundamental cause of glaucoma.

A new technique, which uses microlaser stimulation and microelectrodes, allowed researchers supported by the NEI to stimulate selected nerve cells in individual circuits in this dense meshwork of cells. Results from this approach show how individual nerve cells function together. Previously, these results were very difficult to obtain because of the inaccessibility of these functional units. Further refinement of this approach could lead to the development of a powerful tool for isolating discrete networks of nerve cells and for learning how they function and organize information.

During fiscal year (FY) 1994, results from a NEI-supported study the Orinda Longitudinal Study of Myopia (OLSM) were published suggesting a possible genetic link to myopia. Measurements were made in more than 700 school children ages six to 14 on each child's refractive error, corneal curvature, crystalline lens power, and axial ocular dimensions. Parents reported their own vision status. The investigators found that school children whose parents are nearsighted have differently shaped eyeballs than children whose parents are not nearsighted. Children of myopic parents had longer eyes even before the onset of myopia. These results suggest that the premyopic eye in children with a family history of myopia already resembles the elongated eye present in myopia. The OLSM will continue to provide a wealth of information on ocular and refractive development in children in the years ahead.

These are highlights of a few of the research accomplishments of the intramural and extramural laboratory and clinical scientists supported by the NEI during FY 1994. More detail on these and other accomplishments in the field of vision research can be found in the reports and project descriptions contained in this annual report. It is a pleasure to provide this recognition of the researchers' efforts in addressing the visual health needs of our Nation.

Carl Kupfer, M.D.
Director
National Eye Institute

EXTRAMURAL RESEARCH

Report of the Associate Director for Extramural Research

Jack A. McLaughlin, Ph.D.

Research activities supported by the extramural Vision Research program address the leading causes of blindness and impaired vision in the United States, including retinal diseases, corneal diseases, cataract, glaucoma, strabismus, and amblyopia. The program seeks to increase understanding of the normal development and function of the visual system; to understand the causes of and to better diagnose, prevent, and treat sight-threatening conditions; and, to enhance the rehabilitation, training, and quality of life of individuals who are partially sighted or blind.

In working to this end, the Vision Research program supports vision research through grants, cooperative agreements, and research and development contracts; encourages high quality clinical research, including clinical trials and other epidemiologic studies; encourages research training and career development in the sciences related to vision; sponsors scientific workshops in high-priority research areas to encourage exchange of information among scientists; and carries out a construction, alteration, and instrumentation program of grants for public and private nonprofit vision research facilities.

For FY 1994, an estimated total of $248,425,000 was expended for NEI extramural grants, cooperative agreements, and research and development contracts in the following categories and amounts:

Research Grants	$233,541,000
Research Training Awards	$7,294,000
Research and Development Contract	$7,590,000
Total Extramural Support	**$248,425,000**

The following sections highlight some of the recent accomplishments of NEI-supported investigators.

Division of Basic Vision Research

Peter Dudley, Ph.D., Director

Retinitis Pigmentosa

The major diseases of the retina affect primarily the photoreceptor cells and the neighboring tissue called the pigmented epithelium. The photoreceptors are fragile and easily damaged in the face of hereditary defects, aging, toxic agents, overexposure to light, and dietary deficiencies. They are targets for such diseases as RP and AMD, which are leading causes of blindness. The basis for understanding these diseases lies in gaining fundamental knowledge of the molecular machinery and cellular organization of these specialized cells. Structural information, combined with detailed biochemical and physiological knowledge, sets the stage for a molecular dissection of genetic diseases. Recent identification of defects in photoreceptor-specific genes in some inherited retinal diseases support this concept.

RP is one of the most common human inherited eye disorders, causing blindness from degeneration of the rod and cone photoreceptors in the retina. Patients with RP develop night blindness and loss of midperipheral vision early in the disease. As the disease progresses, a shrinking island of central vision remains resulting in "tunnel vision." Unfortunately, many patients are blind by middle age. In the United States, RP affects 50,000 to 100,000 people of all races. RP exhibits heterogeneity, meaning that different mutations in a person's genetic material or DNA cause similar clinical symptoms. There are two forms: "allelic heteogeneity," refers to different mutations within a single gene (rhodopsin for example), and "locus heterogeneity," which refers to gene defects at different chromosome locations. RP can be transmitted as an autosomal dominant, autosomal recessive, or X-linked trait.

The understanding of RP, currently an incurable retinal degeneration, has progressed recently with the discovery of a number of genetic mutations in important photoreceptor proteins. In about one-quarter of the cases of RP, mutations have been identified in the genes for rhodopsin, peripherin/RDS, rod phosphodiesterase-β subunit, and the cyclic guanosine monophosphate (cGMP)-gated channel. Although most cases are considered to be monogenic, *i.e.*, in one particular family only one chromosome locus is thought to be defective, Dr. Dryja, from the Harvard Medical School, has demonstrated an unusual inheritance pattern in three families with RP called "digenic inheritance." Mutations in two genes, the unlinked photoreceptor-specific genes-ROM1 and peripherin/RDS, were found to be responsible for this particular form of RP. The interesting feature of this unusual form of inheritance is that although variability in disease severity was observed in patients with other mutations (*e.g.*, rhodopsin), the complete lack of clinical symptoms in some persons with a peripherin/RDS gene mutation, had not been observed. The explanation for this was revealed when a mutation in ROM1 was discovered in patients who also had the peripherin/RDS mutation resulting in disease expression.

Norrie's disease (ND) is a rare X-linked hereditary eye disease characterized by congenital blindness. Investigations so far show the disease-causing gene to be located on the X chromosome. Genetic analysis has pinpointed a chromosome site that may be the actual gene. Four separate mutations have been identified that cause deletions of part of the chromosome housing the apparent ND gene. But formal conclusive evidence that the ND gene is involved in the disease awaits identification of more gene mutations in ND patients.

Dr. Wong, from Duke University, has discovered a new mutation in the ND gene of a male infant that results in loss of a small part of the ND protein. The normal ND protein, which appears to function in retinal and brain development, contains 11 cysteine amino acids. The point mutation discovered by Dr. Wong results in a loss of two cysteine residues. The discovery of another separate point mutation increases the list of mutations in the ND protein and appears to confirm the importance of the full complement of 11 cysteines in its function. This discovery may result in a useful diagnostic test to confirm an initial clinical determination of ND.

Glaucoma

In the United States, about two million people have glaucoma, but, because of the insidious nature of the disease, many are unaware of its presence. Additionally, about five million Americans, some of whom will develop glaucoma, have elevated intraocular pressure (IOP). Primary open-angle glaucoma (POAG) is the most severe form of the disease and is most common in people older than age 60. Approximately 80,000 people with this form of the disease will become blind. African Americans are affected disproportionately from POAG with a risk factor five times that of the white population for people older than age 40. The rate for blindness due to POAG in African Americans is six times higher than that of the

rest of the population, reflecting a more severe disease.

The mechanism by which the optic nerve is damaged by glaucoma is not known. The relative influence of genetic and environmental factors is also unclear. However, there is some hope for understanding the cause of the disease because juvenile onset glaucoma, a form of the disease characterized by early adulthood onset and elevated IOP, displays an autosomal dominant pattern of inheritance. This mode of inheritance makes a genetic approach ideal for the study of this disease.

Dr. Stone, from the University of Iowa, Dr. Richards, from the University of Michigan, and Dr. Wiggs, from the Tufts New England Medical Center, have identified a number of families with sufficient numbers of individuals with glaucoma so that it is now possible to perform genetic linkage studies. To date medical histories have been collected from families in Michigan, New England, and Iowa. The ultimate goal is to identify a "glaucoma gene." Recently, one disease-associated gene has been mapped by linkage analysis to chromosome 1. Corroborating data from different laboratories using different families have confirmed this location. Linkage analysis has placed the gene to within approximately a 20 to 80 gene region on the chromosome.

A possible association between juvenile onset glaucoma and POAG may lie in identifying a causal factor for elevated IOP, be it a block in the aqueous humor outflow pathway or some other as yet unidentified factor.

Keratoconus of the Cornea

Keratoconus is a progressive condition characterized by noninflammatory thinning and protrusion of the cornea, leading to its cone-shaped appearance. Keratoconus has an incidence of about five in 10,000 in the general population, and approximately 100,000 keratoconus patients require eye care in the United States annually. Most of these patients need multiple contact lens fittings during their life and 10 to 20 percent ultimately require a procedure called a penetrating keratoplasty to correct their condition. Recent retrospective studies suggest that keratoconus is the leading cause for cornea transplantation in the United States.

Dr. Rabinowitz, from the Cedar-Sinai Medical Center, has been examining the genetic basis for keratoconus. He is using a technique called videokeratography to obtain an accurate assessment as early as possibleof the chances for developing this condition. Pilot studies on patients with advanced disease indicated three distinguishing features: central corneal steepness, nonsymmetric steepness when comparing the superior and inferior corneal regions, and large central refractive differences between the right and left corneas. These features were quantitatively indexed and applied to broader family studies. This work suggests an autosomal dominant mode of heredity with variable expressivity.

Dr. Rabinowitz's group is exploring three independent approaches to identifying a keratoconus gene(s); cytogenetic studies have been initiated. These have not yet been informative, although there is a report in the literature that Angelmans' syndrome patients with keratoconus as one of their ocular findings carry a deletion on the long arm of chromosome 15. Family pedigree studies have been initiated, using videokeratographic data. To date 200 normal subjects, 110 keratoconic patients, and 210 family members have been analyzed and have donated blood for future genetic studies. Enrollment is approximately 60 percent complete, and, so far, more than 70 family pedigrees have been constructed. These include several large families with multiple affected members in at least four generations. Analysis will be performed within the year to determine the genetic mode of inheritance. Longitudinal candidate gene studies are under way in one of the large multigenerational families. Some specific genes involved in the synthesis of collagen have been excluded. Other collagen genes and the genes for enzymes involved in collagen metabolism are being explored.

Acquired Immunodeficiency Syndrome

As many as one-third of adults with AIDS eventually develop neurological symptoms, including problems with memory, coordinated movement, and sensation. These problems can occur in the face of almost complete absence of direct infection of nerve cells or neurons by the HIV-1. Recently, loss of neuronal cells in the brains and retinas of AIDS patients has been observed and is thought to contribute to the neurologic and retinal dysfunction. Retinal ganglion cells involved in the transmission of information from the retina to the brain appear to undergo physical changes in these patients.

What are the mechanisms that cause the observed changes in retinal ganglion cells in AIDS patients? Dr. Lipton, from Children's Hospital in Boston, has been studying this problem. There appears to be growing scientific evidence that for infections like HIV, which lead to the injury of neurons, a complex web of interactions between cells of the immune system and neurons is involved. Dr. Lipton has been studying one particular protein involved in this system called gp120.

Gp120 is a protein present on the outer surface of the HIV virus and is associated with increased intracellular concentrations of the ion calcium (Ca^{++}). When gp120 is added to retinal ganglion cells growing under laboratory conditions, the cells are injured. How does this happen? In general, when cells take up Ca^{++} in an uncontrolled fashion, they die. Dr. Lipton has shown that there is a certain kind of receptor for the neurotransmitter glutamate that is apparently involved in this process. Glutamate is actively taken back into cells after release as a neurotransmitter so that it can be reused. Apparently gp120 can hinder this "reuptake." Recently, Dr. Lipton has discovered a novel antagonist to the glutamate receptor that may give protection from gp120 neurotoxicity. In addition to its affect on glutamate, gp120 can also cause macrophages—cells that can swallow up and destroy bacteria and foreign materials—to release substances toxic to neuronal cells.

Retinal Neuroscience—Molecular Basis of Signaling

Vision begins when cells in the retina called rods and cones capture light and initiate a series of biochemical events that send an electrical message to the brain. Rod cells respond to dim light. The red-, green-, and blue-sensitive cones are sensitive to bright light and give us color vision. The orchestrated interplay of molecules in photoreceptors is called signaling or visual phototransduction and describes the way light is converted to an electrical signal destined for the brain.

Light, which is focused by the cornea and the lens, enters the retina as energy particles called photons, which are absorbed by rhodopsin. Attached to rhodopsin is a small molecule, vitamin A, which changes its molecular configuration slightly when struck by a photon. This change in configuration or isomerization is the initial event in a process called the transduction cascade, which now becomes rapidly amplified as rhodopsin collides with many molecules of transducin. Transducin also has a smaller molecule attached to it. In this case, it is not a vitamin but a nucleotide called guanosine triphosphate (GTP). The binding of GTP to transducin is a consequence of the initial contact with rhodopsin. Transducin then dissociates into separate parts, one of which binds to yet another molecule called phosphodiesterase (PDE), activating it. PDE, an enzyme, actuates the triggering event in phototransduction—the conversion of cGMP into an altered form called simply GMP to denote the fact that it is no longer a circular molecule. This hydrolysis or splitting of cGMP closes a molecular gate in the membrane of the rod cell resulting in a decreased flow of small ions like sodium into the cell. This change in membrane potential of the cell generates an electrical signal to the neighboring retinal cells and then to the brain for signal decoding.

Current research has now turned to establishing the complete molecular basis of signaling in visual cells, signal termination, and adaptation. Knowledge of the structure of

each of the components of the phototransduction cascade is necessary to understand the mechanisms behind the dynamics of, and potential sites for, regulation of signaling in visual cells.

The electrical events that initiate vision begin with the capture of light that leads to the closing of cGMP-controlled membrane ion channels. These ion channels directly control the flow of ions across the outer cell membrane. Rods and cones use similar but not identical molecular components to transduce light into neural signals to the brain, having their own distinct visual pigments as well as enzymes necessary for phototransduction. In general, although cones appear to have a similar phototransduction scheme to rods, the exact mechanisms underlying their low sensitivity and quick response to light remain unclear. To study the basis for these differences Dr. Molday in Vancouver, British Columbia, has cloned the cGMP-gated channels from rod and cone photoreceptors of the chicken retina and examined their molecular and electrical properties. Using a special technique to engineer the cells to express specific molecules, he demonstrated that chicken rod and cone cells each synthesize different forms of cGMP-gated channels.

The characterization of the rod channel protein is of great scientific interest because of its central involvement in phototransduction. Dr. Yau, from The Johns Hopkins School of Medicine, has cloned a protein from human retina that has about one-third similarity in structure to a similar protein from bovine retina. By itself, the human-derived protein does not self-associate to form a functional working channel. However, when combined with the channel protein isolated from bovine retina it functions characteristically like the native channel. What does this mean? It implies that the newly discovered human protein is but one of a group of different proteins that when assembled together form the native channel protein. It is a clue that the channel protein may be made up of different subunits, a characteristic shared by other channel proteins that are controlled by small molecules like cGMP.

At the end of a signaling event, the molecules involved must return to their original state. A key feature in the recovery and reactivation of phototransduction is the involvement of the ion calcium in Ca^{++}-sensitive regulation of guanylyl cyclase (GC), an enzyme that synthesizes cGMP. During visual transduction, closure of the cGMP-gated channels reduces Ca^{++} influx, while efflux by Na^{+}, K^{+}, and Ca^{++} continues, causing a decrease in Ca^{++} within the cell. This fall in Ca^{++} stimulates GC and allows recovery of the sensitivity to light. Dr. Palczewski, from the University of Washington, has shown that the regulation of GC is mediated by a novel photoreceptor-specific protein. The cloning and characterization of this molecule have shown there are three areas on the molecule that can bind Ca^{++}, which suggests it has features in common with a large family of molecules called calcium-binding proteins. In addition, Dr. Hurley, from the University of Washington, has shown that human photoreceptors use similar physiologically relevant proteins in responding to and recovering from light.

Human color vision involves three separate light-capturing molecules, called the red, green, and blue photopigments, to designate the wavelength of light to which they are most receptive. Although the sequence of amino acids for the red and green pigments are identical, except for 15 of their 365 amino acids, their light absorption characteristics differ significantly. In an extensive study using a technique called mutagenesis to selectively and individually alter specific amino acids, Dr. Oprian, from Brandeis University, has determined that there are only seven amino acids responsible for the light absorption differences between the red and green pigments. The mechanisms by which these amino acids determine the light absorbing properties of the photopigment are unknown. However, they do indicate how sensitive biological molecules can be to small changes in amino acid composition. These changes can have health implications when the change or "muta-

tion" leads to disease. A good example is RP, where certain mutations in the gene coding for rhodopsin result in disease. On the other hand, some mutations in the gene coding for rhodopsin can manifest themselves only in an altered sensitivity to wavelengths of light with no impact on quality of life.

The physical structure of the biologically active subunit of rod cell transducin was described recently by Dr. Hamm, from the University of Illinois. Her research suggests how "nucleotide exchange" might occur in phototransduction so as to induce changes in the surfaces of proteins to activate them and explain a mechanism for GTPase activity not evident from previous studies. Dr. McKay, from Stanford University, has determined the crystal structure of recoverin, a recently discovered member protein that serves as a calcium sensor. Ca^{++} plays a critical role in the recovery phase of visual excitation and in adaptation to background light. The light-induced lowering of Ca^{++} in retinal rod outer segments appears to function in system recovery after a light response. With the recent discovery that calcium-bound recoverin prolongs the photoresponse, most likely blocking the phosphorylation of photoexcited rhodopsin, the information obtained on the crystal structure will lead to further insights about recovery and adaptation in vision.

Corneal Angiogenesis

The growth of new capillary blood vessels, neovascularization or angiogenesis, occurs during many normal ocular processes such as wound healing and embryonic development. However, angiogenesis can also be a component of serious eye pathologies such as diabetic retinopathy, corneal clouding following infection or traumatic injury, and neovascular glaucoma. The presence of blood vessels in the cornea makes it much more difficult for a transplanted cornea to survive and remain clear.

Current treatment of corneal neovascularization relies on the use of topical steroids. Unfortunately, these agents have significant side effects, including immunosuppression, osteoporosis, stomach ulcers, and diabetes. Dr. Proia, from Duke University School of Medicine, and Dr. Schwartzman, from the New York Medical College, are developing new categories of angiostatic drugs to inhibit new vessel growth with a more favorable therapeutic profile. Recent advances include the discovery of a novel class of angiostatic steroids with fewer side effects. An independent family of angiostatic substances, like the cytochrome oxidase P450 inhibitors flurbiprofen and clotrimazole, has also been recently described. These drugs decrease corneal inflammation and neovascularization by affecting the levels of prostaglandins synthesized during the inflammatory process.

Lens Development

Lens formation begins with lens epithelial cell division and proceeds with differentiation into fiber cells. Fiber cells are terminally differentiated cells that are essentially "sacs" of proteins devoid of cellular structures. Continuous differentiation of epithelial cells into fiber cells is required to maintain lens transparency. Any disruption of the internal controls that balance growth and differentiation has implications for cataract formation.

Determining the signals that control epithelial cell division and differentiation into fiber cells has been an area of active investigation. Using model systems, Dr. Paul Overbeek, from the Baylor College of Medicine, has shown the importance of growth factors in this process. Growth factors are a diverse group of small molecules that can trigger cell division. A challenge to lens biologists is to determine which of the many known growth factors are responsible for cell differentiation. Scientists have identified a number of potentially important growth factors including insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF), and a novel factor found in the vitreous—lentropin. Dr. Overbeek has been studying these growth factors by putting the genes for their expression into a mouse and creating what is called a transgenic mouse so that one can assign a functional role to

various growth factors. Overexpression of IGF-I and FGF in the lens of transgenic mice leads to congenital cataracts, substantiating the importance of these molecules in lens development and providing scientists with a model for cataract formation.

Dr. DePinho, from the Albert Einstein Medical School, and Dr. Greep, from the University of Wisconsin, are interested in how lens development is controlled. Because fiber cell formation is essential for maintenance of lens transparency, preserving the balance between cell division and cell differentiation is critical. Recently, the retinoblastoma (Rb) and protein(p)53 tumor suppressor genes have been shown to play a role in eye development. Transgenic mice in which the normal functioning Rb gene is knocked out demonstrate a condition called microphthalmia (small eyes). In these mice, lens cell DNA synthesis proceeds unimpeded while cell elongation and differentiation is inhibited. Continuous epithelial cell division is incompatible with normal lens functioning. To avoid this problem it appears that p53 acts as a backup regulatory gene. Thus, in the absence of Rb activity, p53 is expressed causing apoptosis or programmed cell death and resulting in the microphthalmic condition.

Neural Processing of Visual Information

Understanding visual processing and its disorders requires knowledge of the human nervous system, including molecular, genetic, chemical, cellular, and integrative processes that underlie perception and the control of eye movements. As this knowledge increases it will help research aimed at preventing or treating disorders such as strabismus (misalignment of the eyes), amblyopia (commonly known as "lazy eye"), myopia, and neuro-ophthalmological disorders.

Although disorders of visual processing may not always cause total blindness, they may seriously diminish the quality of life of those they afflict. Because these conditions affect more than 10 percent of the population, they constitute serious public health problems. Continued advancement of clinical investigation in this field rests upon an improved understanding of basic visual mechanisms.

Development

Using a new technique to trigger activity in selected nerve cells in a circuit, Dr. Katz, from Duke University, has shown that developing visual circuits are quite different than anatomical studies alone would indicate. Katz and his colleagues are using an approach he developed called "laser photostimulation" or "caged glutamate" to show how the wiring in the mammalian visual system changes during development.

The nervous system is a dense network of many individual nerve cells that communicate with each other at specialized gaps called synapses by chemical messengers called transmitters. The sending cell releases the transmitter at the synapse and the receiving cell responds if it has an appropriate receptor for the transmitter. Currently, Dr. Katz is exploring the visual circuits in the brain that use one common transmitter, glutamate. Thin slices of the nerve tissue are bathed in glutamate, which is "caged"—each molecule is trapped within a surrounding molecule that renders it inactive. A tiny electrode is inserted inside a single nerve cell and the surrounding tissue is bombarded with minute spots from an ultraviolet laser. This bombardment releases the glutamate from its chemical cage causing that single nerve cell to become active. This gives researchers information on how the surrounding neurons functionally interact.

Initial results indicate that the visual system does not produce large numbers of neurons that are subsequently pruned during development. Instead, there is more of a redistribution of synapses in a circuit rather than an elimination during development. Of greater significance for the field of neuroscience, however, is that this method promises to become a powerful tool to trace how networks of brain cells function and organize information.

Dr. McConnell, from Stanford University, is investigating how the highly organized cellular patterns and connections among nerve cells in the adult visual cortex arise during development. When these nerve cells are "born," they receive instructions to leave the site of their origin and migrate long distances through a complex environment to other sites in the brain where they acquire a new identity. Dr. McConnell wants to determine if an individual nerve cell "knows" who it is during its development. This is done by transplanting young nerve cells (whose normal fate she can predict) from one brain to another and then allowing the behavior of the transplanted nerve cell to reveal its "commitment" to its normal identity. What has been found is that by the time they are born, nerve cells seem to "know" exactly who they are. In fact the progenitor cells, or mothers of the young cells, actually make decisions about the fates of the progeny, and they do so in a way that is exquisitely sensitive to the local environment in which the young nerve cells are generated.

Dr. McConnell has been trying to find out how the progenitor cells generate different types of nerve cells at specific times in development. It appears that the progenitors cannot make the right choice without information from neighboring cells. The progenitor cells are either sending signals to one another or receiving them from other nerve cells, which provide instructions that are essential for normal fate determination. Currently, studies are aimed at trying to identify the molecules and genes that are involved in this process. This research will provide us with insights into how the complex connections of the brain are formed and how inappropriate connections are established when this process is flawed.

Personal Guidance System for the Visually Impaired

Drs. Loomis and Klatzky, researchers from the University of California, Santa Barbara, are developing a "personal guidance system" that "shows" the environment to blind users. The users wear stereo earphones mounted on glasses through which they receive information that tells them how to navigate through an unfamiliar environment.

The system, housed in a backpack, picks up signals from the global positioning system (GPS) that was developed for the military and is transmitted from satellites. The system integrates this positional information with a geographic information system (GIS) in the form of a computerized map to create a "virtual acoustic display" that the user perceives as a talking map where preprogrammed objects and landmarks announce themselves as words through the earphones at the appropriate time and volume to cue the user to their precise location as he or she navigates. This provides the user with information on distance and direction for successful navigation through an unfamiliar environment. The device relies on triangulation of signals from GPS satellites to determine precise location. The information is transmitted to the system's on-board computer that contains a digitized map. The user wears an electronic compass that tells the system exactly where he or she is. The system then generates speech that the user perceives as coming from the location of the object or landmark in the real world. This allows the user to preview a trip permitting a rehearsal of a planned walk. Other workers are developing similar systems that use the GPS, but this is the first one to use a virtual acoustic display. Dr. Loomis and his colleagues plan to miniaturize the system further to make it less bulky and easier to carry. A cane or a guide should still be used with this system to inform the user of any obstacles that are not on the computer map. When the personal navigation system is fully implemented, it promises to expand the mobility of blind persons for navigating through unfamiliar areas and significantly improve their ability to carry out every day tasks.

Division of Collaborative Clinical Research

Richard Mowery, Ph.D., Director

The Division of Collaborative Clinical Research plans and directs a program of grant, cooperative agreement, and contract support for applied clinical vision research, including clinical trials, natural history studies, surveys, cohort studies, and case-control studies. Currently, the division manages 19 clinical trials, 17 epidemiology projects, three eye health education demonstration projects, and 10 analysis and planning projects with an annual budget of $38.6 million.

Following are highlights of some of the major findings from these research projects.

Retinal Diseases

Macular Degeneration

AMD is the leading cause of legal blindness in the United States for persons older than age 60. Despite the major public health significance of AMD, the cause of the disease is not understood and major risk factors, other than age, have not been determined. For instance, a case-control study of risk factors for AMD was conducted on Long Island, New York, among individuals ages 50 to 79. Patients with "wet" (exudative) AMD and patients with "dry" (nonexudative) AMD were compared with individuals without the disease. An increased risk for both AMD types were associated with current smoking, light eye-color, family history of AMD, poorly controlled hypertension, and certain nutritional factors. This study indicates that many causative factors may contribute to the disease.

Beneficial treatment effects of laser photocoagulation for some of the people with the neovascular form of the disease have been reported for the Macular Photocoagulation Study. However, laser photocoagulation does not restore severe vision loss due to the disease, and some people lose vision despite treatment.

Animal research and preliminary epidemiologic studies suggest a protective role for certain micronutrients in the development of both cataract and macular degeneration. In particular, vitamins and certain trace minerals with antioxidant capabilities are being studied for their role in age-related vision loss. Currently, published studies are intriguing but are not conclusive in establishing a role for antioxidant nutrients in the development or worsening of AMD or cataract.

The Age Related Eye Diseases Study (AREDS) has as one of its goals to evaluate the effect of high-dose antioxidant vitamins and zinc on the progression of macular disease and lens changes. Patients with minor to severe drusen pathology and minimal lens opacities are being enrolled in the AREDS, randomized to a high-dose dietary supplement or placebo, and followed for a minimum of seven years to assess the progression of both eye disorders. Because patients enrolled will not have optically significant lens opacities, this study will also provide information regarding the risk factors for cataract formation. The prospective nature of this study, its focus on the progression of drusen pathology, and its evaluation of the effects of high-dose supplements make this a unique and much needed study. Eleven clinical centers, a coordinating center, a photographic reading center, a drug distribution center, a central laboratory, and the provider of the supplements—American Cyanamid/Storz-Lederle—participate in the study. Patient enrollment and randomization are ongoing.

Diabetic Retinopathy

A well-coordinated public health approach to diabetic retinopathy requires accurate data regarding the incidence and associated risk factors for the disease. Diabetes is a major cause of visual impairment and blindness in the United States. Results from a 10-year population-based cohort study conducted in southern Wisconsin recently reported the 10-year incidence rate of blindness (visual acuity of 20/200 or worse) in diabetic persons was 1.8 percent, 4.0 percent, and 4.8 percent in

younger-onset, older-onset taking insulin, and older-onset not taking insulin, respectively. This rate of blindness increased with age and with duration of diabetes. Respective 10-year rates of visual impairment (visual acuity of 20/40 or worse) were 9.4 percent, 37.2 percent, and 23.9 percent. For all three diabetic groups, macular edema or more severe retinopathy was associated with greater visual loss. Also, visual loss was associated with smoking (younger-onset group) and increased systolic blood pressure (older onset group taking insulin). These data indicate visual loss is common in diabetic populations and identify several modifiable risk factors for visual loss due to diabetes. The data also assist in the estimation of costs for diabetic care.

Retinopathy of Prematurity

Retinopathy of prematurity (ROP) is a serious public health problem among low-birth-weight infants. The NEI supported a multicenter trial of Cryotherapy for Retinopathy of Prematurity, which demonstrated that cryotherapy reduces by approximately one-half the risk of unfavorable retinal and functional outcome from threshold ROP. Cryotherapy, although helpful, is expensive, ablates a significant portion of the retina, is not always successful, and its long-term sequelae are unknown.

Despite the highly significant advances in the treatment of ROP by cryotherapy, ROP continues to affect an increasing number of very low-birth-weight survivors. The NEI is currently supporting the Supplemental Therapeutic Oxygen for Prethreshold Retinopathy of Prematurity (STOP-ROP) Study. The STOP-ROP is a multicenter, controlled clinical trial initiated to determine if supplemental therapeutic oxygen will reduce the proportion of infants with prethreshold retinopathy of prematurity who advance to threshold ROP. In addition to ophthalmic outcomes, this study will obtain neonatal outcome information, including data on growth, ventilatory stability, chronic lung disease, neurological maturation, and length of hospital stay. Recruitment for this study began in early 1994 and is being conducted at 20 clinical centers in the United States. In addition to the support being provided by the NEI, the Institute has arranged collaboration and support for this study from the National Institute for Child Health and Human Development (NICHD) and the National Institute for Nursing Research.

Cytomegalovirus Retinitis

Cytomegalovirus (CMV) retinitis is by far the most crucial ocular problem for people with AIDS who live more than one year. A potentially blinding disease of the retina, CMV retinitis affects between 25 and 30 percent of people with AIDS. Through the Studies of the Ocular Complication of AIDS (SOCA), a network of investigators with expertise in AIDS clinical research, retinal disease, and clinical trial methodology, the NEI has expedited the testing of treatments for CMV retinitis. These investigators demonstrated the safety and efficacy of foscarnet for the treatment of CMV retinitis. Foscarnet was also shown to extend the life expectancy of people with AIDS by approximately four months compared with patients who took ganciclovir. All patients with CMV retinitis relapse when anti-CMV therapy is discontinued. Therefore, continuous maintenance therapy is required. Even on continuous maintenance therapy, all patients with CMV retinitis eventually relapse.

Although relapse can often be controlled, with each relapse, additional retina is destroyed. Therefore, treatment strategies designed to prolong the time to relapse are needed. The SOCA investigators are currently comparing the use of combination therapy using foscarnet and ganciclovir with standard therapy to see if it will extend the time to relapse and disease progression. Other studies that are being designed will use newly developed oral compounds and implant devices to look at safety and efficacy issues, extension of relapse time, plus the improvement in quality of life.

Glaucoma

Epidemiology

Glaucoma is a major cause of visual impairment in the African-American populations. African Americans have an earlier age of onset of the disease compared with Caucasians, suffer blindness at a greater rate, and may be more resistant to treatment. Unfortunately, few well-designed epidemiologic studies have examined the prevalence rate of glaucoma in African-American populations and limited information exists on risk factors for the disease.

The Barbados Eye Study is a population based-prevalence survey that was recently conducted among 4,709 residents of Barbados ages 40 to 84. The prevalence of glaucoma was found to be seven percent. Male gender and a family history of glaucoma were associated with the disease. Open-angle glaucoma was diagnosed by strict definition, including both visual field and optic disc criteria. This minimum, conservative prevalence estimate of glaucoma was high, particularly among those older than age 50. If study results are extrapolated to the population of Barbados, the prevalence of glaucoma is one in 11 among persons older than age 50, one in nine in persons older than age 60, and increases to one in six in persons older than age 70. Comparison of this study with that conducted in 2,395 urban African Americans and 2,913 urban Caucasian residents of Baltimore, indicates the age-adjusted prevalence of glaucoma in Barbados is 1.5 times higher than that in the Baltimore African-American population and 7.1 times higher than in the Baltimore Caucasian population. As such, a higher prevalence of glaucoma in African-Caribbean populations compared with African-American populations is found and these differences raise the question of possible genetic factors.

The contribution of genetic factors to glaucoma are planned for investigation in a study designed to address familial aggregation of the disease. The surviving parents, siblings, spouses, and children (older than age 40) of 100 Barbados participants with glaucoma will be examined for eye disorders, and risk factor information will be obtained. This information will increase our understanding of the inheritance of glaucoma and have direct clinical implications in identifying those family members at high risk for developing the disease.

A report of family history from the Baltimore Eye Survey, a population-based prevalence survey conducted among 5,308 African-American and Caucasian residents of east Baltimore as a risk factor for glaucomas, has been published. Glaucoma was diagnosed in 161 participants in this survey. Participants in this survey were interviewed to determine if first-degree relatives (parents, siblings, and children) had glaucoma. Results indicated a higher risk of glaucoma in siblings than in parents. However, these estimates may be biased because glaucoma was self-reported, not diagnosed, and prior knowledge of glaucoma diagnosis among participants influenced their recall of affected individuals.

Treatment

Currently, the most common treatment for glaucoma is the use of medications to lower pressure in the eye. Although the medications have been demonstrated to be very effective at lowering pressure, the impact of lowering pressure on visual field loss is not completely understood. The economic effect of glaucoma treatment with medications is substantial, accounting for millions of dollars in yearly expenditures in the United States alone. Furthermore, these pressure-lowering medications can often have significant side effects and require the patient to adhere to a strict administration schedule, thus potentially having a profound effect on a patient's quality of life.

The NEI is currently supporting three new clinical trials designed to evaluate treatment strategies for ocular hypertension and glaucoma. The Ocular Hypertension Treatment Study is designed to determine whether medical reduction of intraocular pressure prevents or delays the onset of visual field loss and/or

optic disc damage. Recruitment of patients began in early 1994 at 21 clinical sites throughout the United States. Fifteen hundred ocular hypertensive subjects, at least 500 of whom will be African Americans, are being randomly assigned to either close observation or to a stepped medical regimen. Participants will be followed for a minimum of five years.

The Early Manifest Glaucoma Trial is a randomized, controlled clinical trial designed to determine whether and to what extent reduction of intraocular pressure (IOP) influences the course of chronic open-angle glaucoma. Investigators at the University of Lund in Malmo, Sweden, collaborating with investigators from the State University of New York at Stony Brook, are studying 300 patients with newly diagnosed glaucoma. Participants are being randomized to receive pressure lowering treatment or observation with no or delayed treatment. Both groups are followed closely with computerized perimetry and fundus photography. Recruitment of patients began in 1993 and will continue for an estimated two to three years. Followup of patients will be conducted for approximately four years.

Filtration surgery can also be effective in controlling IOP. When successful, filtration surgery may provide the most effective long-term, consistent control of IOP with the least likelihood of requiring supplemental medications. The Collaborative Initial Glaucoma Treatment Study is designed to compare the efficacy of a medical regimen with filtration surgery as the initial treatment for newly diagnosed glaucoma. This clinical trial will compare the two treatment strategies in terms of controlling IOP and will specifically investigate the impact of these treatment strategies on the participants quality of life. Recruitment of the 600 patients began in November 1993. The study is being conducted at 11 clinical centers located throughout the United States.

Corneal Diseases

Prospective Evaluation of Radial Keratotomy Study

Approximately 25 percent of adults in the western world have myopia. Some of these people may be candidates for radial keratotomy (RK), a procedure aimed at correcting or reducing myopia through surgical incisions made in the cornea. The NEI has been supporting a multicenter controlled clinical trial designed to evaluate the short- and long-term safety and efficacy of a single standardized technique of RK.

The five-year followup results from the Prospective Evaluation of Radial Keratotomy (PERK) Study indicated that RK was safe and that few very serious complications resulted from the procedure. However, it was difficult to predict the outcome for an individual eye. In addition, the refractive error continued to change in some patients over time.

In 1994, the PERK investigators completed 10-year followup examinations of patients who were enrolled in the PERK Study. Data from these examinations will soon be available and will be one of the few sources of information on the long-term stability of RK.

Strabismus, Amblyopia, and Visual Processing

Optic Neuritis and Multiple Sclerosis

More than one-half of all people with first-time optic neuritis, a vision-impairing inflammation of the optic nerve that affects more than 25,000 Americans each year, will eventually develop multiple sclerosis. Multiple sclerosis is a debilitating disease of the central nervous system that affects as many as 500,000 Americans. Based on data from two years of followup of patients enrolled in the Optic Neuritis Treatment Trial, researchers found that treating first-time optic neuritis patients with a combination of intravenous and oral

corticosteroids lowers their risk of developing multiple sclerosis. The results from this research, published in *The New England Journal of Medicine,* offer the first scientific evidence that intravenous corticosteroids help to delay the progression of multiple sclerosis. It also suggests that this treatment may provide similar benefits for people with not only optic neuritis but other early symptoms of multiple sclerosis.

Myopia

Results from an NEI-supported study published in the *Journal of the American Medical Association* suggest a genetic link to myopia. Researchers from the University of California, Berkeley, School of Optometry report on 716 school children ages six to 14 who are enrolled in the OLSM. Measurements were made on each child's refractive error, corneal curvature, crystalline lens power, and axial ocular dimensions. Parents reported their own vision status. The investigators found that school children whose parents are myopic have different shaped eyeballs than children whose parents are not nearsighted. Children of myopic parents had longer eyes even before the onset of myopia. These results suggest that the premyopic eye in children with a family history of myopia already resembles the elongated eye present in myopia.

The OLSM will provide a wealth of information on ocular and refractive development in children in the years ahead. Future results may provide the eye care provider or pediatrician with the answer to the question frequently asked by myopic parents— "What are the chances that my child will develop myopia?"

Ocular Injuries

Ocular trauma is a major cause of monocular blindness in the United States and, in 1986, was responsible for estimated hospital costs of up to $200 million. Self-reports of lifetime ocular injuries were obtained in the population-based Baltimore Eye Survey of adults older than age 40. Ocular injuries were reported by 14.4 percent of participants (n=5308); men reported a greater number of ocular injuries than women. The number of injuries were similar among both African-American and Caucasian men, however, visual consequences of injuries were more severe among African-American men.

DIVISION OF BIOMETRY AND EPIDEMIOLOGY

Report of the Acting Director, Division of Biometry and Epidemiology

Roy C. Milton, Ph.D.

The Division of Biometry and Epidemiology (DBE) is made up of a Clinical Trials Branch, an Epidemiology Branch, and a Biometry Section. Dr. Roy Milton is the Acting Director for the division. Drs. Frederick Ferris III and Robert Sperduto serve as chiefs of the two branches, respectively; Dr. Roy Milton is the head of biometry.

The DBE has three main functions: research, education, and consultation. Research is the dominant function. It is the division's mission to plan, develop, and conduct human population studies concerned with the cause, prevention, and treatment of eye disease and vision disorders, with emphasis on the major causes of blindness. This includes studies of incidence and prevalence in defined populations, prospective and retrospective studies of risk factors, natural history studies, clinical trials, genetic studies, and studies to evaluate diagnostic procedures.

The DBE carries out a program of education in biometric and epidemiologic principles and methods for the vision research community. This program consists of courses, workshops, a fellowship program for ophthalmologists, publications, and consultation and collaboration on research.

The DBE provides biometric and epidemiologic assistance to the NEI intramural and extramural staff and to vision research workers elsewhere. The assistance ranges from consultation to collaboration as coinvestigator.

Research Highlights

The Eye Disease Case-Control Study

One of the diseases studied in the Eye Disease Case-Control Study was idiopathic macular holes, a disease by which women are more affected than men. Results from this study showed that higher fibrinogen levels and a history of glaucoma were associated with an increased risk of the condition. The use of exogenous estrogens was associated with a decreased risk. The fibrinogen finding was unexpected, and it is not clear whether this is a chance finding or whether higher levels of fibrinogen can increase susceptibility to the forces of vitreous traction, perhaps by compromising the macular blood supply or by some yet unexplained mechanism.

Within this large case-control study, a substudy was conducted to evaluate the relationships between dietary intake of carotenoids and vitamins A, C, and E and the risk of neovascular macular degeneration, the leading cause of irreversible blindness among adults. A higher intake of dietary carotenoids was associated with a lower risk. Among the specific carotenoids, lutein and zeaxanthin were most strongly associated with a reduced risk of this form of macular degeneration. Several food items rich in carotenoids were inversely associated with macular degeneration. In particular, a higher frequency of intake of spinach or collard greens was associated with a substantially lower risk for AMD.

The Framingham Offspring Eye Study

Eye examination data from 1,086 parents examined in the Framingham Eye Study and 896 offspring examined in the Framingham Offspring Eye Study (FOES) were used to study familial associations for nuclear, cortical, and posterior subcapsular lens opacities. For any pair of siblings, if one sibling had a nuclear opacity the odds of the other sibling having such an opacity were estimated to be more than triple. Similar findings were noted for posterior subcapsular opacity. The strong associations between siblings for nuclear and posterior subcapsular opacities suggest there is clustering of lens opacities within families. The clustering may be due to genetic or environmental factors.

The Italian-American Natural History Study of Age-Related Cataract

The Italian-American Natural History Study of Age-Related Cataract has estimated the incidence and progression of cortical, nuclear, and posterior subcapsular opacities in a large followup study. The three-year cumulative incidence for persons ages 65 to 74 years (the largest group studied) was 18 percent, six percent, and six percent for cortical, nuclear, and posterior subcapsular opacities. Progression was much higher than incidence for each type of opacity. The study suggested that patient age, baseline lens status, cataract grading system, definition of change, and analytic methodology may have important effects on estimates of cataract incidence and progression.

The Early Treatment Diabetic Retinopathy Study

Recent publications from the Early Treatment Diabetic Retinopathy Study (ETDRS) multicenter, randomized clinical trial include an evaluation of the effect of aspirin versus placebo on mortality and morbidity from all causes and specifically from cardiovascular disease. This study is one of two primary prevention studies of the effect of aspirin on cardiovascular disease and is the only large study to include women. The results are similar to the studies on males without diabetes and demonstrate that aspirin use reduces the risk of cardiovascular disease.

Previous ETDRS publications had not reported any increase in the occurrence of vitreous/preretinal hemorrhages in study patients assigned to aspirin. With new data suggesting the importance of aspirin use in persons with diabetes who are at risk for cardiovascular disease, it was important to investigate this possible risk of aspirin use in more depth. Data published recently (*ETDRS Report No. 20*) demonstrate that the severity and duration of these hemorrhages were not significantly affected by the use of aspirin and that there were no ocular contraindications to its use in persons with diabetes who require it for treatment of cardiovascular disease or for other medical indications.

Research Activities

Clinical Trials

The Early Treatment in Diabetic Retinopathy Study

The ETDRS was designed to determine when to use photocoagulation for diabetic retinopathy. Patients with macular edema, preproliferative retinopathy, and mild or moderate proliferative retinopathy were studied. Various treatment strategies of focal and scatter (panretinal) photocoagulation were compared with no photocoagulation. In addition, the study evaluated the placebo-controlled effects of daily administration of aspirin on the incidence of microvascular and macrovascular complications. The study also investigated factors associated with the progression of disease.

Recruitment was completed in March 1985 with the enrollment of 3,711 patients. In December 1985, the study reported that focal photocoagulation of clinically significant diabetic macular edema substantially reduces the risk of visual loss. It was further reported that focal treatment increases the chances of

visual improvement, decreases the frequency of persistent macular edema, and causes only minor visual field losses. Subsequent reports indicated that whether treated early with scatter photocoagulation or followed closely and treated as soon as they reached the high-risk stage, all eyes had low rates of severe visual loss. Scatter photocoagulation is not recommended for eyes with mild or moderate nonproliferative diabetic retinopathy, provided careful followup can be maintained. When retinopathy is more severe, scatter photocoagulation should be considered and usually should not be delayed if the eye has reached the high-risk proliferative stage.

Sixteen ETDRS reports have been published and additional manuscripts are being prepared. Drs. Lloyd Aiello and Frederick L. Ferris, III serve as cochairmen, Dr. Richard L. Mowery is project officer, and Dr. Emily Y. Chew serves as a member of the analysis planning group. The results of effects of aspirin on vitreous hemorrhage in patients with diabetes mellitus have been accepted for publication. A report of accommodation in the ETDRS population has been submitted for publication. Analyses in progress include the association of serum lipids and retinal hard exudates, risk factors for severe visual loss, risk factors for development of high-risk proliferative diabetic retinopathy, fibrovascular proliferation associated with macular edema, and causes of severe visual loss. In collaboration with Dr. Thomas Gardner, from Hershey Medical Center, results of analyses of digoxin and retinopathy have also been submitted for publication.

In addition, patients with mild to proliferative retinopathy are being followed with extensive psychophysical testing in the NEI Clinical Center, to determine the mechanisms for loss of visual acuity in diabetic retinopathy. An additional study of long-term followup of diabetic retinopathy following laser photocoagulation is under way at the NEI Clinical Center.

The Krypton-Argon Regression of Neovascularization Study

The Clinical Trials Branch began the Krypton-Argon Regression of Neovascularization Study (KARNS) in three pilot clinics in December 1983. The major objective of this randomized clinical trial is to compare red krypton laser with blue-green argon laser panretinal photocoagulation for treating neovascularization on the optic nerve head caused by diabetic retinopathy. Twenty-nine new clinics were enrolled in KARNS starting in August 1984. At the termination of the study in June 1990, a total of 1,063 patients had been randomized. This study is unique for the NEI because the functions for both the coordinating center and the fundus photography reading center are being handled by staff of the Clinical Trials Branch. Another feature of this multicenter trial is that the participating clinics receive no financial reimbursement from the NEI for their participation. Drs. Ferris and Chew direct this study along with Dr. Lawrence Singerman. KARNS *Report No. 1* (*Ophthalmology*, 1993) showed the two treatments were equally effective in arresting neovascularization of the disc; additional analyses are under way.

The Linxian Eye Study

The NEI joined an ongoing NCI-supported clinical trial of nutrition and cancer in north central China in 1991 to determine whether the vitamin/mineral dietary supplements administered in the Linxian Cancer Trials for the preceding five years have affected the risk of age-related cataract and AMD. Eye examinations were conducted in 1991 on 5,390 members of the Linxian Study cohort. Dr. Sperduto is project officer, and the project team includes Drs. Milton and Chew from DBE and Dr. Tian-Sheng Hu, an ophthalmologist from Beijing. Findings for cataract have been published. Analyses are being conducted for the macular degeneration component.

The Italian-American Trial of Age-Related Cataract

A new collaborative Italian-American clinical trial is being planned to study the effect of a broad spectrum vitamin/mineral supplement on the risk of age-related cataract. Twelve hundred people will be randomized to either the supplement or a matching placebo and followed for five years. Recruitment into the study will begin in early 1995. The study will complement the ongoing AREDS, which is being conducted in the United States. Dr. Sperduto is the project officer and Dr. Milton is a member of the project team.

Intramural Program Clinical Trials

Drs. Ferris and Chew and Ms. Remaley are collaborating with Dr. Nussenblatt on four additional randomized clinical trials in the NEI Intramural Program of the Clinical Center: (a) a trial of a sustained-release intraocular drug delivery system for ganciclovir therapy of cytomegalovirus retinitis in patients with AIDS; (b) a trial to evaluate the efficacy of a heparin-surface modified intraocular lens in reducing the incidence and severity of postoperative inflammatory episodes following extracapsular surgery in uveitis patients with cataracts; (c) a trial of anti-inflammin, a peptide, in the treatment of anterior uveitis; and (d) a trial of oral S-antigen or retinal extract versus placebo in patients with uveitis.

Other

Dr. Ferris now represents the NEI on the Data Monitoring Committee of the United Kingdom Prospective Diabetes Study, a clinical trial of alternative treatment regimens in the management of patients with diabetes. Followup is scheduled to continue in this study until 1997.

Epidemiology

The Age-Related Eye Disease Study

The AREDS is designed to collect natural history data of 4,600 patients of ages 55 to 80 years with bilateral drusen of different types or with unilateral advanced AMD. This study will evaluate the rates of development and progression of AMD, the rates of visual loss due to retinal lesions of AMD, and the risk factors associated with the development and progression of AMD. Evaluation of lens change over the 10-year AREDS study period will provide an opportunity to evaluate factors associated with the development of cataracts. In addition, a clinical trial will be performed to determine whether antioxidants (vitamin C, E, and beta-carotene) and zinc can prevent the development or retard the progression of AMD and cataract. There are 11 clinical centers, a photographic reading center, a central laboratory, and a coordinating center. Identification of study participants began in September 1990. In November 1992, participants were evaluated with qualifying visits, and participants were randomly assigned to the study medications beginning in February 1993. Dr. Ferris, chairman; Dr. Sperduto, director of lens project; and Dr. Chew are directing the scientific aspects of the AREDS; Dr. Natalie Kurinij is project officer.

The Eye Disease Case-Control Study

The Eye Disease Case-Control Study is designed to identify risk factors for neovascular macular degeneration, idiopathic branch retinal vein occlusion, idiopathic central retinal vein occlusion, rhegmatogenous retinal detachment, and idiopathic macular hole. Dr. Sperduto is the study chairman, Ms. Rita Hiller is director of data analysis, and Dr. Chew is a member of the project team. Two papers were published this year: risk factors for idiopathic macular hole and the effect of dietary carotenoids and vitamins A, C, and E on neovascular age-related macular degeneration.

The Diabetes in Early Pregnancy Study

Dr. Chew and Ms. Remaley, in collaboration with Dr. James Mills of the NICHD, examined the effects of pregnancy on diabetic retinopathy in the Diabetes in Early Pregnancy Study. Data collection terminated in 1985, and a manuscript has been submitted. Further analyses on the effects of pregnancy in proliferative retinopathy are planned.

The Italian-American Natural History Study of Age-Related Cataract

The Italian-American Natural History Study of Age-Related Cataract was designed to estimate the rates of development and progression of the different types of lens opacities and the associated risk factors. Dr. Sperduto is the project officer; Dr. Milton and Ms. Remaley are members of the project team. Three papers were published this year, including one that provides estimates of the rates of cataract development and progression. Analyses of risk factors for cataract development and progression are under way.

The Framingham Offspring Eye Study

Dr. Sperduto is the project officer, Dr. Milton is the alternate project officer, and Drs. Podgor and Freidlin and Ms. Hiller are members of the project team for FOES. This study is designed to examine familial relationships for age-related cataract and AMD among parents examined in the Framingham Eye Study (1973 to 1975) and their children examined 1989 to 1991. Dr. Podgor has used generalized estimating equation methodology in the analyses of these data. A manuscript describing the study's findings for cataract has been accepted for publication. A manuscript describing the association of opacities between and within eyes of individuals has been submitted. Analyses of macular degeneration are planned.

Other

A manuscript has been accepted for publication on risk factors for strabismus, using data from the NICHD Collaborative Study and in collaboration with Dr. Mark Klebanoff, from the NICHD. The DBE project team includes Drs. Chew, Tamboli, Zhao, and Podgor and Ms. Remaley. Esotropia developed in three percent and exotropia in 1.2 percent of the children followed for seven years. Esotropia was more common in Caucasians than in African Americans. The occurrence of exotropia was similar in the two races. Maternal cigarette smoking during pregnancy and low birth weight were independent and important risk factors for both esotropia and exotropia. Analyses are under way on sibling association in strabismus and on risk factors for congenital cataract.

Drs. Valerie Freidlin and Marvin Podgor continued to provide consultations with NEI Clinical Center investigators, especially for various studies of measurement of lens opacities. Dr. Freidlin is collaborating with Dr. Ellwein in management and analysis of Medicare data on ophthalmologist services.

Statistical Methods

Dr. Podgor and Dr. Joseph Gastwirth, from the George Washington University, collaborated in an investigation of the use of scores for stratified data. A paper has been accepted for publication. Drs. Podgor, Gastwirth, and Cyrus Mehta, from Cytel Corporation and Harvard University, have proposed methodology for efficiency robust tests for ordered 2xK contingency tables. A paper has been submitted.

Ongoing Activities

Members of DBE are active in consultations and educational and professional activities, including referees for professional journals, associate editors or members of editorial boards, members of data and safety monitoring committees for clinical trials, training of staff fellows, invited and contributed presentations at professional society and other meetings, advisory committees for grant-supported cooperative agreements, and technical advisors to the World Health Organization (WHO).

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OFFICE OF INTERNATIONAL PROGRAM ACTIVITIES

Report of the Acting Assistant Director for International Program Activities

Terrence Gillen, M.A., M.B.A.

The mission of the NEI is to reduce the prevalence of blindness, visual impairment, and eye disease worldwide through basic and applied research and training. Although excellent ophthalmic procedures and eye-care delivery systems are accessible in the developed world, adequate health care is not readily available in all parts of the developing world. This widening gap in visual health between developed and developing nations threatens to have ominous consequences. If present trends continue, the number of blind people—estimated at 24 million—will more than quadruple during the next 40 years. As many as 90 percent of these blind people will live in developing countries.

This large-scale disablement caused by blindness is not only a costly obstacle to economic development, it is also a catastrophic loss of human potential in the areas of the world most desperately in need of a healthy workforce. In addition, because more than 80 percent of all cases of blindness can be considered avoidable—that is, they could have been prevented or could be cured using available and locally appropriate technology—such deprivation is a truly needless denial of a basic human right for millions of people. Therefore, the NEI undertakes international activities to facilitate the development and application of effective prevention and intervention programs. These efforts are coordinated by the Institute's Office of International Program Activities (OIPA), which was created in February 1989. The OIPA enhances NEI's international programs by:

- Evaluating available health technologies, promoting the most cost-effective intervention and prevention programs, and encouraging their availability for affected populations, especially in developing countries.

- Conducting collaborative applied research studies to develop preventive methods for treating specific eye diseases.

- Conducting controlled clinical evaluations of promising research findings.

- Exchanging information on recent scientific advances and their appropriate application to visual problems.

The NEI supports international research on six blinding diseases that have a major worldwide effect: cataract, onchocerciasis, ocular toxoplasmosis, glaucoma, diabetic retinopathy, and vitamin A deficiency.

Highlights of Recent Scientific Advances Resulting From International Activities

Because cataract is responsible for about one-half of the developing world's curable blindness and is a major problem for the United States as well, the NEI has developed a collaborative research program that includes projects to prevent blindness from cataract with collaborating groups in Italy, India, and Latin America. In addition, health services research expertise from the NEI is made available to

selected collaborating partners through training activities and the conduct of joint research projects.

For example, intramural scientists from NEI's Laboratory of Mechanisms of Ocular Diseases (LMOD) in collaboration with colleagues at the Centre for Cellular and Molecular Biology in Hyderabad, India, are studying aging-related modifications to lens crystallins. These scientists have demonstrated that chemicals present in smoke, either from tobacco products or from wood fires, can directly damage lenses in organ culture studies. In addition, molecular geneticists in the NEI's Section on Cataract have initiated gene linkage studies with scientists at Osmania University and the L.V. Prasad Eye Institute in Hyderabad on selected families with hereditary cataract.

The collaborative Italian-American Study of the Natural History of Age-Related Cataract has completed a four-year followup study of cataract. Objectives of the natural history study were to estimate the rates of development and progression of the various types of lens opacities, identify risk factors associated with the development and progression of cataracts, and evaluate cataract classification schemes. A manuscript describing study results has been accepted for publication in the *American Journal of Ophthalmology.*

Summary of International Programs and Activities

Country-to-Country Activities

Barbados

Open-angle glaucoma is the leading cause of blindness in African Americans and is a major cause of visual impairment and disability. The incidence of glaucoma has not been measured precisely in any population, and the risk factors related to its development are largely unknown. Since 1988, the Barbados Eye Study has examined more than 4,200 persons ages 40 to 86 years as part of a population-based study to determine the prevalence and risk factors for glaucoma and other eye disorders such as diabetic retinopathy, AMD, cataract, and visual impairment. In 1992, the Barbados Incidence Study was initiated to estimate the incidence of glaucoma and other ocular disorders in individuals in the Barbados prevalence survey who were free of disease. In addition, risk factor analysis will be conducted to identify associations with development of glaucoma and to characterize those who have progressive eye disease. (See "The Barbados Eye Study: Prevalence of Open-Angle Glaucoma" in the June 1994 issue of *Archives of Ophthalmology,* 112 (6): 821-829.)

Brazil

In collaboration with the U.S. National Institute of Allergy and Infectious Diseases (NIAID), NIH and three Brazilian scientific organizations in Sao Paulo—Escola Paulista de Medicina, Clinica Erexim, and Laboratory Fleury—the NEI developed a research program on the immunology, basic mechanisms, and epidemiology of toxoplasmosis in southern Brazil. The prevalence of ocular toxoplasmosis in this population was found to be more that 30 times higher than previous estimates for the same condition elsewhere. In this population, ocular toxoplasmosis appears to be a sequela of postnatal rather than congenital infection. Studies performed in 1993 on postnatal blood from newborns in southern Brazil have shown a low percentage of immunoglobulin M positivity, further suggesting that the disease in southern Brazil is acquired.

India

The NEI and the Indian Council of Medical Research (ICMR) have developed a collaborative blindness research program under the 1983 Indo-U.S. Science and Technology Initiative. This program includes projects to reduce blindness from vitamin A deficiency, cataract, and Eales' disease in India. Indian government funds for the work come through the ICMR, and U.S. Government funds are provided through the National Science Founda-

tion and the NEI. In addition, the NEI collaborates with Indian scientists under the U.S.-Indo Subcommission program.

The NEI director, deputy director, and special advisor to the director participated as consultants to the World Bank to develop a proposal by the Government of India for an initiative in cataract blindness control. Technical meetings have been held in New Delhi and Madurai to provide the knowledge base on which training and surgical guidelines can be developed for a twofold expansion of cataract surgery, with explicit attention to the quality and extent of vision restoration.

Intramural scientists from the NEI, LMOD are collaborating with colleagues at the Centre for Cellular and Molecular Biology on studies on aging-related modifications to lens crystallins. Cataracts typically occur at an earlier age and are more heavily pigmented in the Indian population than in the U.S. population. In an attempt to elucidate the molecular mechanisms underlying this difference in color, the scientists are comparing over a wide range of ages the fluorescence spectra for normal intact lenses from the Indian population with age-matched Eye Bank lenses from the U.S. population. The Indian population lenses have significantly greater amounts of pigmented fluorescent compounds than do the U.S. population lenses. These compounds may play a direct role in cataractogenesis through their ability to function as photosensitizers.

In organ culture studies, collaborating investigators have demonstrated that chemicals present in smoke, either from tobacco products or from wood fires, can directly damage lenses. A primary site of damage appears to be the cell membrane. Epidemiological studies have indicated that smoke-derived compounds are probable risk factors for cataract. These studies will continue in an effort to identify the pathological mechanisms involved. A paper describing the studies to date has been submitted for publication.

Molecular geneticists in the NEI, Section on Cataract have initiated gene linkage studies with scientists at Osmania University and the L.V. Prasad Eye Institute in Hyderabad on selected families with hereditary cataract. The prevalence of consanguineous marriages in this region of India greatly increases the likelihood of recessive cataract phenotypes. Blood samples from individuals in suitable pedigrees are being shipped from India to the NEI for linkage analysis. A geneticist from Osmania University, who was trained at the NEI in relevant techniques, has returned to Hyderabad to establish a laboratory so that much of the work can be performed in India. In one family the cataract trait has been linked to a particular chromosome and a potential candidate gene has been identified.

In September, Dr. Prem Prakash, chief of the Dr. Rajendra Prasad Centre for Ophthalmic Sciences, a component of the All India Institute of Medical Sciences, visited the NEI to discuss possible future research collaboration.

Italy

The Collaborative Italian-American Study of the Natural History of Age-Related Cataract has completed a four-year followup study of cataract. Objectives of the natural history study were to estimate the rates of development and progression of the various types of lens opacities, identify risk factors associated with the development and progression of cataracts, and evaluate cataract classification schemes. A total of 1,297 patients participated in the followup study. Data collection lasted from April 1989 to April 1994. Organizations participating in the study included the Institute of Ophthalmology at the University of Parma, the Laboratory for Epidemiology and Biostatistics at the Istituto Superiore di Sanita in Rome, and the NEI in the United States. A manuscript describing study results has been accepted for publication in the *American Journal of Ophthalmology*.

Investigators at the University of Parma and NEI are also collaborating in a study to determine whether the complete or partial deletion of the glutathione-S-transferase I gene

is an important risk factor in the development of senile cataract. Blood has been drawn from approximately 300 cataract patients and is now being analyzed to determine complete or partial gene deletion at the University of Parma and the NEI's LMOD.

A new collaborative Italian-American study is being planned to evaluate the effect of multivitamin supplements on the risk of cataract development and progression. Approximately 1,200 subjects will be randomized to either a multivitamin/mineral supplement or matching placebo and followed for five years. Professor Giovanni Maraini, from the University of Parma, and Dr. Robert Sperduto, from the NEI, will be the study's principal investigators. The Laboratory for Epidemiology and Biostatistics, Istituto Superiore di Sanita, Roma, and the DBE at the NEI serve as the study's Coordinating Centers. The seven-year study was scheduled to start in late 1994.

Mexico

An international collaboration has been established by scientists in the NEI, LMOD, Section on Cataract, to investigate the relationship between enzyme deficiency diseases and cataract. For example, a candidate gene study was initiated to determine whether a deficiency in sorbitol dehydrogenase in a family where several members have congenital cataracts is due to changes in SDH gene structure or expression. This study is possible through the cooperation of the Unidad de Investigacion Biomedica y Hospital de Pediatria, Instituto Mexicano del Soguro Social, Guadalajara, Mexico.

Sweden

Many eye diseases, especially retinal degenerations, could be successfully treated if human retinal transplantation were possible. In animal models, visual cells that have been transplanted do not develop and function normally. However, a new differentiating factor has been discovered and is being expressed using molecular biology techniques at the NEI. This factor, which is a protein that causes neuronal-like differentiation, is being tested *in vitro* by NEI collaborators in Sweden at the University of Gothenburg and the University of Lund to determine if it will cause retinal cell differentiation. The ultimate purpose of these investigations is to develop cells that could be transplanted into the human eye *in vivo* and function normally.

In collaboration with protein biochemists at the Karolinska Institute in Stockholm, NEI cataract researchers are investigating the evolutionary relationships of ζ-crystallin, an enzyme/crystallin of certain species, with other oxido-reductases. Establishing such relationships with enzymes of known function should help to identify the physiological roles of ζ-crystallin in the lens and in other tissues where it is present at low levels. A paper reporting these analyses is being prepared.

The Early Manifest Glaucoma Trial is a randomized, controlled clinical trial to determine whether and to what extent reduction of IOP influences the course of chronic open-angle glaucoma. Investigators at the University of Lund are collaborating with investigators at the State University of New York at Stony Brook and will study an estimated 300 patients with newly diagnosed disease. Participants will be randomized either to pressure-lowering treatment or to observation without treatment. Both groups will be followed closely with computerized perimetry and fundus photography. Recruitment of patients began in 1993 and will continue for an estimated two years. Followup of patients will be conducted for four years.

United Kingdom

The UK Prospective Diabetes Study is a prospective randomized study of different therapies to determine whether improved blood glucose control or improved blood pressure control of noninsulin-dependent diabetes will reduce morbidity and mortality. The study began in 1977 and has recruited more than 5,100 newly diagnosed diabetic patients. Patients who fail to respond to diet therapy are randomized to diet therapy or active

therapy with sulfonylurea, insulin, or metformin. As part of the study, hypertensive diabetic patients have been randomized to tight blood pressure control (with either an angiotensin-converting enzyme inhibitor or β-blocker) or to less tight control. The development and progression of diabetic retinopathy in these patients are being assessed by retinal photography. The study is completing 11 years of patient followup.

Activities With International and Multinational Organizations

During the past year, the NEI has supported investigations of blinding eye diseases that have a worldwide effect. These studies are implemented through bilateral agreements between foreign countries and the United States; other types of country-to-country programs such as those supported by U.S. Agency for International Development; and collaborative activities with the WHO, the Pan American Health Organization, foundations, and private and voluntary organizations such as the Lions Clubs International.

The NEI is continuing to provide technical advice to Lions Clubs International in the development of its $100 million SightFirst initiative, a global sight conservation program aimed at substantially reducing the prevalence and incidence of preventable and curable vision loss.

In FY 1994, the NEI continued its activities as a WHO Collaborating Center for the Prevention of Blindness. The NEI director continues to serve on the WHO's Special Advisory Panel in the Prevention of Blindness. Other NEI staff members have, on request, consulted to the WHO program.

The NEI is working closely with nongovernmental organizations in designing service and research programs to reduce the prevalence of blindness, regardless of its etiology, throughout the world. A special emphasis last year and in the next few years will be an evaluation of program performance in selected countries.

Extramural Programs

In FY 1994, NEI granted 14 awards to foreign institutions in six countries. Research and training projects were supported in lens and cataract, glaucoma, visual system development, photoreceptors, phototransduction, visual cortex, visual abnormalities, Leber's disease, nutrition of the eye, ocular complications of diabetes, and the prevention of blindness. Awards covered both basic and clinical research projects.

Intramural Programs and Activities

The NEI continues to serve as an international center for research and training on eye disease. In FY 1994, 25 visiting fellows, 21 visiting associates, 17 visiting scientists, 19 special volunteers, and three guest researchers from more than 20 countries conducted research at the NEI facilities in Bethesda, Maryland. Their work included basic laboratory investigations on the molecular structure and development of the visual system, sensory and motor disorders of vision, and the biochemical bases of retinal and corneal diseases and cataract development. In addition, visiting scientists collaborated with NEI investigators in clinical studies to define, treat, and prevent vision disorders, such as genetic and developmental defects, ocular inflammatory disease, and ocular complications due to systemic conditions such as diabetes.

Office of Science Policy and Legislation

Report of the Associate Director for Science Policy and Legislation

Michael P. Davis, M.S.

The Office of Science Policy and Legislation is responsible for program planning, analysis, and evaluation activities; development and maintenance of a computerized management information system; and legislative and other program coordination activities. In addition to the activities listed below, the office had one organizational change during the year. The Scientific Reporting Branch was made an office within the Office of the Director and is now called the Office of Health, Education, and Communication (OHEC).

Policy, Legislation, Planning, and Evaluation Branch

Carmen P. Moten, Ph.D., Chief

During FY 1994, the Policy, Legislation, Planning, and Evaluation Branch provided numerous reports concerning research-related activities of the NEI. Specific activities included the preparation of recurring and *ad hoc* program analyses in response to requests from the NIH, The U.S. Public Health Service (PHS), and the U.S. Department of Health and Human Services (DHHS); serving as the focal point on program planning, analysis, evaluation, and legislation; and planning, coordinating, carrying out, and monitoring NEI program evaluations. Principal activities for this branch are specified below:

- Preparation of NEI *Scientific Advances— FY 1995 Congressional Justification.*

- Preparation and submission for the *Annual Report of Aging-Related Eye Disease Research.* Eye diseases and disorders, such as AMD, cataract, glaucoma, and diabetic retinopathy, are causes of blindness and visual impairment among older Americans.

- NEI submission for the *Survey on Nursing Research and Related Activities Supported by NIH,* FYs 1989 to 1992.

- Response to the Fogarty International Center (FIC) concerning the NIH report to the Senate on *Biodiversity.*

- NEI submission on *Gene Therapy and Hereditary Rare Diseases.* Much of the molecular genetics research that is conducted by NEI researchers is instrumental in developing the basic understanding necessary to pursue gene therapy treatment strategies for rare ocular and visual system hereditary disorders.

- NEI submission for the top scientific accomplishments in basic research, applied research, and clinical trials.

- Review of various PHS FY 1996 Legislative Proposals from the Health Resources and Services Administration and the Food and Drug Administration. The NEI felt compelled to respond to the legislative proposal Regulation of Human Tissue for Therapeutic Use. The proposal intends to provide a regulatory program for banked human tissues that addresses the fundamental differences between human tissues used for medical products. Fees will also

be charged for registration, operating permits, and inspections to support the cost of the program. The NEI believes that such fees could threaten human eye tissue transplantation. Almost all, if not all, eye banks of the Eye Bank Association of American are 501(c)3 organizations. They depend heavily on philanthropic contributions in providing eye tissue for research or for patient care. Most rural and small eye banks would have serious difficulty with the imposition of fees, given the difficulties of meeting current operation budgets.

- NEI submission for supported projects that are evaluating cigarette smoking as a potential risk factor for eye diseases—Healthy People 2000 Tobacco Priority Area.

- NEI submission for the *1994 NIH Disease Prevention Annual Report.* The report highlighted basic research, applied research and clinical investigation, intervention studies, and professional and public education.

- NEI submission report to the Office of the Director (OD), Office of Disease Prevention for the *Fiscal Years 1992 and 1993 Prevention Outlays and Full Time Equivalent Positions.*

- Report to the OD, Office of Science Policy and Technology for the *1994 Minority Health Legislation.* The NEI supports three demonstration projects aimed at developing, implementing, and evaluating comprehensive culture-specific and community-based education programs for the prevention of diabetic retinopathy.

- *NEI Planning Activities Report,* in response to the OD, Office of Strategic Planning and Evaluation request.

- NEI submission for the *NIH Legislative Implementation Plan—NIH Revitalization Act of 1993.*

- NEI submission for the *FY 1996 NIH Plan for HIV-Related Research.*

- Analyses of draft materials from the PHS Office of Disease Prevention and Health Promotion for editorial review of the *Guide to Clinical Preventive Services* report of the U.S. Preventive Services Task Force.

The branch also has been involved in researching, writing, and editing various reports requested by the NIH, PHS, DHHS, Congress, and nongovernmental organizations and individuals, including the following:

- NEI submission for the *NIH Intramural Research Program* to the Subcommittee on Appropriations Health and Education, Congressmen Louis Stokes.

- Report to the Schepens Eye Research Institute on the NEI extramural funding for various eye diseases and disorders.

- NEI submission for the *1995 White House Conference on Aging.* The mandate of such conferences is to produce recommendations for aging policy to span the next decade.

- Report of the NEI director for the *1993-1994 Biennial Report of the NIH.* The report described research accomplishments, outlined future opportunities, and assessed important policy issues.

- NEI submission for the *Diabetes Mellitus Interagency Coordinating Committee (DMICC) Annual Report.* The report included recent activities of the structure and function of polyol pathway enzymes, epidemiology of diabetic retinopathy, and advances in retinal cell biology.

- NEI submission on *Research Activities Related to Space.* The report highlighted two areas of research, ultraviolet radiation on the delicate tissues of the eye, and the structure and function of the vestibular-ocular reflex.

- NEI submission for the *Annual Legislative Weekend of the Congressional Black Caucus Health Braintrust.* The report highlighted epidemiologic studies, clinical trials, National Eye Health Education Program, demonstration projects, and support for minority scientists, institutions, and students.

The following information was submitted to the NEI Financial Management Branch:

- NEI report of *FY 1993 Actual Outlays for Trans-NIH Research Areas.* The report areas included: accidents and injuries, aging and age-related diseases, Alzheimer's disease, arthritis and musculoskeletal disorders, breast cancer, cystic fibrosis, diabetes, diagnostic imaging/diagnostic radiology, drug development, funding for children (0 to 21), gene mapping for both the Human Genome Project and in nonhumans, gene therapy research, health and behavior research, immunology research, infant mortality/low birth weight, kidney diseases, medical rehabilitation research, minority aids, minority health and assistance, neurofibromatosis, neuroscience, nutrition, orphan drugs, prevention, sexually transmitted diseases, sickle cell diseases, smoking and health, space medicine, stroke, vaccine development, vaccine-related, and women's health.

- Total NEI Basic/Applied/Development research by mechanism, in a tabular format, for the Office of Financial Management and Budget Exhibit 44A.

- Written responses to questions submitted by Appropriations Subcommittee members.

The branch contributed support for the Office of Health Education and Communication (OHEC) concerning the NEI grant portfolio. This portfolio included various types of eye disease-related research conducted by specific investigators such as:

- Retinal cell transplantation studies, RP and related diseases, cataract, dry eye, glaucoma, and tissue plasminogen activator.

The branch provided detailed information for various NIH offices, including:

- A description of NEI-outlays for disease prevention research for the Office of Disease Prevention.

- An analysis of all nutrition-related research supported by the NEI for inclusion in the *Human Nutrition Research Information Management Report.*

- NEI submission on *Skin Diseases Activities/Congressional Report.* The report included a table of all skin disease-related research funded by NEI and research activites of the corneal angiogenesis, genetic studies of keratoconus, cloning of a human corneal desmosomal protein, and collaborative activities.

- NEI table submission of all arthritis and musculoskeletal diseases-related research for the *Arthritis and Musculoskeletal Diseases Interagency Coordinating Committee (AMDICC) Annual Report.*

The branch also provided editorial review of a variety of letters, reports, and other narrative materials for other offices within the NEI.

Management Information Systems Branch

David Scheim, Ph.D., Chief

During the past fiscal year, the Management Information Systems Branch (MISB) has upgraded four of its network servers in Building 31 and EPS to Windows NT advanced server, providing improved speed, reliability, and connectivity. MISB initiated and supervised a contract for the extension of its local area network to a Windows NT server and 10 workstations distributed among the NEI

intramural laboratories, allowing access to administrative data and computer support services. This has enabled MISB, for example, to grant intramural offices access to the Status of Funds program, which MISB also provided to additional users in the administrative and executive offices of Building 31. The integrated architecture provided by the migration to the Windows NT network operating system allows all current and any additional users to run this program from one file server location.

The MISB managed the installation of the TAIMS timekeeping system on approximately 20 workstations within the NEI. Bringing this system to full production entailed assistance and problemsolving for users, particularly in the transmission of aggregated data. The MISB also provided similar assistance in the installation of the ATRAIN system for generating training requests and installed an official airlines guide flight scheduling program for the administrative office. The NEI upgraded several users from Word Perfect 5.1 to 6.0 and arranged training for NEI staff to make this transition.

The MISB upgraded five printers to the Hewlett Packard LaserJet 4 series. Eight modems were upgraded from 2,400 to 14,400 bits per second, allowing much faster communications speeds for remote usage of the NEI LAN by NEI staff on travel or working after hours at home. MISB installed two CD ROM drives, primarily for use in software installations and installed a magneto-optical drive for more reliable daily and weekly backups of all network data. The MISB, in addition, has continued to provide support and maintenance for all 95 workstations in Building 31 and EPS, all software used, and the network infrastructure with no contractor support.

The MISB planned and implemented a comprehensive documentation contract for the NEI's microcomputer LAN, associated hardware and software, and all custom-developed systems at a cost of $23,500. This project, when completed in FY 1995, will provide extensive textual documentation of NEI microcomputer systems; cabling diagrams; and online documentation of equipment, processes, and problem resolution procedures accessible to MISB and all NEI staff. Database tables and access systems for the online components of this effort were developed by MISB staff.

The MISB began a joint effort with the NIAID for the development of a client-server personnel tracking system to maintain information on all permanent and temporary employees. Development will be performed by an NIAID contractor, but MISB input and design assistance will be provided in exchange for consideration of particular NEI requirements. If successful, such collaborative efforts will be used for the subsequent development of a procurement tracking system using state-of-the-art software compatible with the NEI's current database platforms.

The MISB evaluated automated grants awards systems in use at NIH and arranged a $14,000 contract for the customization of one such system for use by the NEI Extramural and Collaborative Program. This contract specifies capabilities provided to the NEI on par with those provided to another ICD through a contract costing about $1 million. Most of the work on this contact was completed in FY 1994.

MISB provided extensive enhancements to its grants information systems during FY 1994. Microsoft SQL Server, the database server for all systems, was upgraded to version 4.2 to allow enhanced functionality. JAM and JAM/DBI, the client tools for these systems, were also upgraded to allow additional functionality. The existing NEI snapshot, council letter, and grants coding systems were upgraded for this new environment.

MISB reprogrammed the grants master online update and SCORE coding routines using the JAM client-server front end in which the NEI's other grant modules are developed. It also converted all historical grants and SCORE data into client-server tables, using custom-developed data conversion and checking routines. The NEI's council letter generation program was also modernized into a more

streamlined client-server process using R&R report writer, which, after evaluation of several such tools, was selected as a future reporting platform. These conversions have allowed the older and less reliable Paradox database applications to be completely phased out.

Automated security, login, and usage tracking functions integrated with LAN login were developed by MISB staff. These functions provide automatic login to grants information systems for NEI staff and also automatic tracking of system usage. Between January and September of 1994, a total of 4,198 logins to grants information systems were recorded, not including system testing by MISB staff. Also, daily check and backup procedures for all active NEI databases, which run automatically each night, were implemented through custom programming by MISB.

The NEI's weekly grants update batch procedures, comprising 24 procedures and 100,000 lines of code previously written in Paradox 3.0, were completely reprogrammed by MISB staff in Microsoft SQL Server Transact SQL language, extensively tested, and implemented. The new batch update procedures run more quickly and reliably and contain extensive built-in system checks to automatically halt processing if an error is detected. The reprogramming was performed to consolidate the NEI's grants system by eliminating an obsolete platform, allowing for easier maintenance and future development in the more reliable, state-of-the art, client-server architecture. The new update procedures have run on weekends virtually error-free since mid-1994.

The MISB has continued to provide custom information reports to NEI staff for internal use and public distribution, with 70 new mainframe requests and an increasing volume of microcomputer-based production reports logged for FY 1994, with rapid turnaround achieved in every case. Weekly and monthly reports, as well, continue to be provided. In addition to its own programming efforts, the MISB has continued to support NEI staff in the use of information resources provided by DRG, the NLM, and other sources, including the DRG information system, CRISP, FOCUS, WYLBUR, MEDLINE, Grateful Med, Legislate, the electronic NIH library catalog, Gopher, and other specialized systems.

The MISB has continued to handle a number of IRM functions for the NEI, including its environment and resources report, strategic plan, tactical plan, budget report, and security functions. MISB staff has continued to represent the NEI on a number of NIH-wide committees, including the Office Technical Coordinators and its network subcommittee, the ADP Extramural Programs Coordinating Committee and its steering committee, the Database Technology Task Force, the NIH lead users group, the Campus Users Research Exchange, and the Technical LAN Coordinators Committee.

OFFICE OF HEALTH, EDUCATION, AND COMMUNICATION

Report of the Director of the Office of Health Education and Communication

Judith A. Stein, M.A.

In a reorganization, the NEI has established the OHEC within the NEI, OD. This new office replaces the Scientific Reporting Branch previously part of the NEI's Office of Science Policy and Legislation. The activities of this office include the National Eye Health Education Program (NEHEP); special activities such as the traveling science museum exhibit; dissemination of research results; publications; response to inquiries from public, health professionals, and the media; and advising NEI staff on all aspects of NEI and NIH scientific reporting, knowledge transfer, health education, and press relations.

National Eye Health Education Program

The NEHEP began the development of a diabetic eye disease education program for Hispanics/Latinos with diabetes. Eight focus groups were conducted across the country to learn more about the knowledge, attitudes, and practices of this target audience as related to diabetes and eye health. Groups were conducted with Central Americans in Washington, D.C.; Puerto Ricans in New York City; Mexican Americans in Los Angeles; and Cuban Americans in Miami. An *ad hoc* working group on Hispanic outreach met to provide recommendations to the NEHEP staff on the development of an education program. It is anticipated this program will be launched in spring 1995.

A television public service announcement (PSA) on glaucoma was produced and distributed nationally in September 1994. The PSA stresses the importance of eye examinations for people at risk for glaucoma especially African Americans older than age 40 and everyone older than age 60. In addition, radio and print PSA's on glaucoma and diabetic eye disease were distributed to media outlets reaching target audiences at risk for these diseases.

The Third National Eye Health Education Conference was held in December 1993. The purpose of this conference was to provide an opportunity for the members of 51 organizations in the NEHEP Partnership to share their program and interests and to develop collaborative eye health education programs at the community level.

One idea that originated at the conference was to conduct an awareness campaign on diabetic eye disease during National Diabetes Month in November. In February, the American Diabetes Association (ADA) and the NEI joined forces to coordinate this event. Nine other Partnership organizations offered support to increase awareness among people with diabetes about the importance of an annual dilated eye examination. These organizations will coordinate local activities. A special brochure was developed for November, adapted from the existing NEHEP *Don't Lose Sight of Diabetic Eye Disease* brochure. The ADA will use its 1-800-DIABETES number as a place to call for a referral to an eye care professional and more information on diabetes and diabetic eye disease. The referral program is coordinated with the American Academy of Ophthalmology and the American Optometric

Association. An extensive media campaign will also be conducted to complement local efforts. This includes a video news release, press release, print advertisement campaign, and a radio program targeted to African-American radio stations.

25th Anniversary Program

The NEI celebrated its 25th anniversary with a nationwide public education program to promote the benefits of vision research. The centerpiece of the celebration was the traveling exhibit V I S I O N, which was developed to highlight the sight-saving results of vision research funded through American tax dollars.

V I S I O N premiered in San Francisco, California at the Exploratorium in October 1993. During 1994, it has been displayed at the Museum of Science and Industry in Chicago, Illinois; the Museum of Discovery and Science in Fort Lauderdale, Florida; Union Station in Washington, D.C.; and at the Louisiana Nature & Science Center in New Orleans. An estimated 125,000 people have visited the exhibit to date. It will travel to approximately 13 more cities, including Boston, Massachusetts; Jacksonville, Florida; Houston, Texas; Los Angeles, California; Portland, Oregon; and Seattle, Washington, during the next few years.

In each location where the exhibit is displayed, NEI grantees and local chapters of the voluntary and/or professional organizations plan a series of regional events designed to increase the public's awareness of vision research. These events include public lectures, vision screenings, press conferences, science writers seminars, and educational programs for school age children.

The NEI also is developing a school curriculum program for children in grades four through eight. The program consists of three lesson plans, interactive classroom activities, and previsit and postvisit exercises. Topics covered include the anatomy and physiology of the eye and visual system, common eye diseases and disorders, and eye safety. The program is designed as a supplement to any science or health curriculum and can be used by either a guest speaker (vision researcher or eye care professional), or the classroom teacher.

The curriculum will be pilot tested this fall in several communities throughout the country and will be available in spring 1995. The Association for Research in Vision and Ophthalmology will print and distribute the program to its 11,000 members this November and will highlight it during the association's annual meeting in May. A marketing and promotion plan will be developed to target science and health educators for children in grades four through eight nationwide.

Public Inquiries Program

The OHEC staff responded to more than 16,000 inquiries from the general public, patients and their families, students, health professionals, legislators, and the media in FY 1994, including 14 pieces of controlled correspondence representing five congressional inquiries and a Presidential proclamation. This reflects an approximate six percent increase in inquiries over last year.

To handle the increase in public inquiries more efficiently, the OHEC staff developed standard information packets on commonly requested eye disorders and diseases, including sarcoidosis, blepharitis, RK, RP, and cataract surgery, and more. To assist health professionals find additional materials on specific eye diseases and disorders, staff members prepared listings of the materials found in the Eye Health Education subfile in the Combined Health Information Database. Two new brochures, written in an easy-to-read format, were developed for people at risk for cataract and AMD.

The OHEC staff also handled 38 Freedom of Information requests.

Scientific Reporting

The NEI publication *Clinical Trials Supported by the National Eye Institute* was developed and printed in fall 1993. This publication provides information on 22 extramural and intramural clinical trials supported by the NEI. Each clinical trial description includes the purpose and design of study, patient eligibility criteria, patient recruitment status, results to date, and participating clinical centers.

Office of the Scientific Director

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00122-14 OSD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Anatomical Studies of the Primate Visual System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Francisco M. de Monasterio	M.D., D.Sc.	Medical Officer	OSD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.40	0.40	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves the study of the anatomical properties and organization of cells in the visual system of primates, with emphasis on the retina and the visual cortex. The studies include the pattern distribution of selectively stained cones in the retina of macaques. The results have provided information on the probable retinal circuitry of the blue-sensitive cone pathway of primate retinal cells.

Project Description

Objectives

To study the anatomical properties and neural organization of the primate visual system.

Methods

Retinal histological processing, intravitreal injection of dyes, computer modeling, and spatial statistical analyses of point and area patterns are used.

Major Findings

The anatomical studies of this project were affected once again by further underground construction work in a building just across the street, in front of the laboratory; in addition, the building housing the laboratory underwent structural renovation during most of this period. Such construction affected both microtomy and microphotography, limiting work to the evening or night hours.

The point pattern of blue-sensitive cones selectively stained with tissue-reactive nonfluorescent dyes (Procion black) was examined at various eccentricities of the macaque retina in distortion-free whole mounts. The point pattern was analyzed in terms of its angular structure and disorder using spatial statistical techniques described in earlier work. Comparison of the parafoveal, extrafoveal central retina, and peripheral retina point patterns indicates that the degree of disorder of the pattern increases slowly with eccentricity, from about 18 percent to about 30 percent. This is equivalent to disturbing each point of a lattice of unit area by 0.18 to 0.30 units along a randomly selected azimuth. Despite this disorder, the pattern maintains its regularity with eccentricity, and side-by-side blue-sensitive cones are very rarely seen.

These results indicate that the blue-cone pattern of macaque retina does not follow a lattice distribution and support a previous model for the development of this point pattern, based on an exclusionary hard core surrounded by a probabilistic soft shell.

Significance to Biomedical Research and the Program of the Institute

Information on the anatomical properties of blue-sensitive cones is important not only to the functional properties of these cones investigated in different basic disciplines but also to the clinical research and diagnosis of acquired retinal disease. The data obtained from the eye of diabetic human donors are particularly promising in this respect.

Proposed Course

These studies will be continued.

NEI Research Program

Retinal Diseases—Retinal Neuroscience

PROJECT NUMBER

Z01 EY 00065-17 OSD

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Physiological Studies of the Primate Visual System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Francisco M. de Monasterio M.D., D.Sc. Medical Officer OSD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.60	0.60	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves the study of the physiological organization of neurons of the visual system of nonhuman primates that may serve as a model for the human visual system. Emphasis is given to the spectral and spatial properties and central projections of retinal ganglion cells and cells from the lateral geniculate body and the visual cortex of macaques. Recordings from color-opponent retinal ganglion cells and parvocellular geniculate cells show essentially identical spectral response bandwidths under similar conditions of stimulation. On the basis of these bandwidth data, both types of cells can be grouped in subtypes which show a direct correspondence with specific color-opponent varieties. The bandwidth data essentially show no difference between geniculate and retinal color-opponent cells subserving similar areas of the visual field.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

To study the neural organization underlying the processing of visual information at different levels of the primate visual system.

Methods

Intracellular and extracellular recordings from single neurons, extracellular recordings of mass responses, computer video stimulation, tangent screen chromatic and spatial stimulation.

Major Findings

The single-cell studies of this project were affected once again by further underground construction work in a building just across the street, in front of the laboratory; this work resulted in often violent shaking that defeated vibration-isolation measures. In addition, the building housing the laboratory underwent structural renovations during most of this period.

The response bandwidth of color-opponent ganglion cells and parvocellular geniculate cells was examined with spectral lights in conditions of neutral chromatic adaptation. The same stimulating conditions were used for both cells, which were typically recorded from the same anesthetized animals. As noted in previous work, spectral response bandwidths have specific "signatures" when plotting bandwidth against the wavelength of the peak sensitivity. The average half-bandwidth at half-maximum sensitivity was 24 nanometers (nm) for retinal ganglion cells and 22 nm for parvocellular geniculate cells. Averaged spectral bandwidths of these cells fall in various distinct signature subgroups matching subgroups based on color-opponent response varieties.

No significant differences, either in average half-bandwidth or spectral signature, were found between color-opponent, center-surround geniculate, and ganglion cells. In fact, for neurons subserving the same or similar areas of the central visual field, the geniculate and retinal bandwidth data could not be distinguished from one another. These results provide further support for an essentially one-to-one relationship between these neurons.

Significance to Biomedical Research and the Program of the Institute

Numerous behavioral, psychophysical, and electrophysiological studies show that the visual performance and characteristics of macaques and humans are extremely similar to one another, so that an understanding of nonhuman primate physiology provides a useful animal model for human visual function.

Proposed Course

These studies will be continued.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Structure and Function of Central Visual Pathways.

Publications

de Monasterio FM: Operating system errors in DOS 5. *J PC Tech* 4:30-37, 1993.

de Monasterio FM: Direct access to interrupt handlers in the system kernel. *J PC Tech,* in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00135-22 OSD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemistry of Retina and Pigmented Epithelium in Health and Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Helen H. Hess M.D. Medical Officer (Research) OSD, NEI

COOPERATING UNITS *(if any)*

Laboratory of Chemoprevention, National Cancer Institute (M. Anzano, Ph.D.)

LAB/BRANCH

Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Effects of nutrition, oxidation, and other environmental factors (light intensity or darkness) on incidence and progress of posterior subcapsular opacities (PSO) associated with genetically influenced retinal degeneration are being studied in pink-eyed Royal College of Surgeons (RCS) rats, in which rod photoreceptor outer segment debris accumulates secondary to a phagocytic defect in retinal pigmented epithelium (RPE). Peroxidation in polyunsaturated fatty acids in debris lead to water-soluble toxic aldehydes, detectable in the vitreous and toxic to lens cells and membranes. Dystrophic rats fed a natural ingredient diet (NIH-07) were highly sensitive to retina light damage, beginning at an intensity of 10 to 40 lux, and 27 percent of the rats developed mature cataracts by 5 to 12 months. Rhodopsin bleaching is essential for retina light damage and PSO. *In vitro*, free retinaldehyde has been shown to be a photosensitizer to generate singlet oxygen, an extremely damaging oxidant for both lipids and proteins, and this also may occur *in vivo*.

In RCS rats reared at 10 to 40 lux, a purified diet (AIN-76A) fortified with antioxidants (0.4 percent β-carotene + 0.01 percent BHT) prevented PSO and mature cataracts. A diet containing additional antioxidants (1,000 mg/Kg diet of vitamin C and 150 mg/Kg vitamin E) retarded retinal degeneration during the time the cataracts would have had their onset (23 to 53 postnatal days) if NIH-07 had been fed. Higher concentrations of vitamin E did not show additional retardation of retinal degeneration.

Effects of increasing environmental lighting in incidence of bilateral mature cataracts were studied in pink-eyed RCS rats fed the NIH-07. Incidence of bilateral mature cataracts (BMC) was 5 percent in rats reared in 10 to 40 lux of cyclic light; but was 25 percent in rats reared in 110 lux of constant light; 70 percent in 270 lux of constant light; and 100 percent in 65-day-old rats given 48 hours of high intensity light (7500 lux). After lengthy or intense illumination, occurrence of disturbed meridional rows of lens epithelial cells and posterior nucleated (Wedl) cells pointed to proliferation of germinative zone epithelial lens cells from deoxyribonucleic acid (DNA) damage. At low illumination, damage can be repaired (stationary cataracts and rare BMC). The results are consistent with the hypothesis of PSC causation by DNA damage to lens epithelial cells. Agents that can have this effect include products of peroxidation of polyunsaturated fatty acids, short wavelength radiation (UV, X-rays, β and γ rays) and numerous chemical mutagens such as N-nitroso-N-methylurea (NMU).

Normal albino rats, injected with NMU at a concentration and dosage sufficient to cause breast cancer, developed BMC by 5 months of age. These cataracts were PSC of a more severe nature than ever seen in RCS rats (exposed to excessive light), with abnormally large cells (some binucleate) not only at the posterior pole but encircling the lens. The retinal showed advanced degeneration.

Project Description

Additional Personnel

J. Samuel Zigler, Jr.	Ph.D.	Chief, LMOD, NEI
Joseph J. Knapka	Ph.D.	Nutrition Consultant, Veterinary Research Program (VRP), National Center for Research Resources (NCRR)
Dennis Bernard	M.S.	Nutritionist, VRP, NCRR
Maria Anzano	Ph.D.	Expert, Laboratory of Chemoprevention, NCI

Objectives

This project is designed to study the biochemical and bionutritional relationships among lens, retinal photoreceptors, retina, retinal pigment epithelium (RPE), and biological fluids in health and disease. It also involves exploring the possibilities for slowing the rate of retinal degeneration and preventing lens opacities and mature cataracts, which are often associated with retinal degeneration in rats and humans.

The cataract that develops in the Royal College of Surgeons (RCS) rat is a posterior subcapsular cataract (PSC), a type that makes up about 10 percent of cases of age-related cataract and occurs in 40 percent of cases of retinitis pigmentosa. PSC also is seen in other hereditary retinal diseases and in radiation damage to the lens. It is characterized by genetically damaged lens epithelial cells, which fail to undergo normal differentiation into lens fibers and instead multiply and migrate to the posterior subcapsular region to form an opacity.

PSC and retinal degeneration are produced in rats by the chemical mutagen N-nitrosoN-methylurea (NMU). This agent has been used to produce breast and prostate tumors as well as glial tumors of the brain in rats. Chemopreventive agents have been found to reduce the incidence of breast and prostate cancers in rats using dual agents, one to reduce or halt multiplication of cells, the other to cause differentiation of the cells. An objective will be to test whether these principles can be used to reduce incidence of PSC and retinal degeneration in rats given NMU. The rat C-6 glial cell line, used for the past 30 years, was derived from rat brain glial cells transformed by NMU *in vivo*; several clones were obtained, C-6 being one of the more differentiated. Transformation of lens epithelial cells *in vivo* by NMU potentially could yield a similarly useful cell line.

Methods

The RCS rat is being studied as an animal model of hereditary retinal degeneration that results from a defect in the RPE as well as a type of cataract that is secondary to retinal degeneration. Bionutrition has been used as a tool to combat lipid peroxidation in the RCS rat retina and to prevent water-soluble toxic aldehyde byproducts from reaching and damaging the lens. The RCS rat cataract is not genetic because the mutant gene is expressed not in the lens but in the RPE; it is instead an outcome of environmental risk factors of both internal and external origin. Thus, the RCS rat is a living laboratory, and the cataracts are susceptible to orchestration by varying risk factors and preventive measures.

Defined diets were prepared and fed to congenic affected and unaffected RCS rats in controlled experiments. The diets were fed to young breeding pairs before they produced their first offspring and to their offspring after weaning so that the experimental animals received their diets from conception to date of observation. Clinical findings were recorded after indirect ophthalmoscopic and biomicroscopic slit-lamp examination. Postmortem examination of the eye included dissecting microscopy and light microscopy of stained specimens. At appropriate times, photography was used to record *in vitro* or *in vivo* data. Analytical methods included standard biochemical, fluorometric, and separation procedures. Special environmental lighting conditions were used to determine histopathological effects on the lens and retina.

In collaborative studies with the National Cancer Institute (NCI), the direct acting deoxyribonucleic acid (DNA) alkylating agent NMU was injected intravenously in a dosage sufficient to produce cancer of the breast in female rats. Experimental rats were given chemopreventative agents. "Control" rats we studied were normals injected with NMU or saline; lenses and retinas were examined histopathologically. Eyes were fixed in 10 percent neutral formalin, embedded in plastic, sectioned at 2 μm, and stained with haematoxylin and eosin.

Major Findings

(1) Bilateral mature cataracts in RCS rats. Dystrophic RCS rats fed a natural ingredient diet and reared from birth at a low-light intensity have a 27 percent incidence of mature cataracts (MC) in one year, most of which are unilateral. In a study of incidence of bilateral MC (BMC) in the RCS rat as a function of environmental light intensity, we found that at low-light intensity (10 to 40 lux) only 5 percent of rats had BMC in one year, while the remaining eyes had stationary cataracts (clear lens fibers between the opacity and capsule). In previous studies, when reared from birth in 100 lux of constant light, 78 percent of rats has MC, 28 percent of which were BMC. Rats exposed to higher intensities of light starting at 22 to 28 postnatal days (when debris containing rhodopsin and polyunsaturated fatty acids is maximal) has a higher incidence of BMC: at 270 lux of constant light, 70 percent of rats had MC, 70 percent of which were BMC; and at 7500 lux for 48 hours, 100 percent of rats had MC, 100 percent BMC. We concluded that rats reared in 10 to 40 lux cyclic light had DNA damage that was repaired in the majority of lenses, and BMC were rare. In constant light of higher intensity, repair did not occur and most or all rats had BMC. The results support the hypothesis of cataractogenesis by toxic aldehydes from peroxidized retina lipids, damaged by singlet oxygen generated by the sensitizer retinaldhyde. Such aldehydes (detected in the vitreous) can damage proteins, lipids, and nucleic acids. Occurrence of disturbed meridional rows of lens epithelial cells and posterior nucleated (Wedl) cells pointed to proliferation of germinative zone epithelial lens cells, possibly from DNA damage.

These results are consistent with a hypothesis of PSC causation by DNA damage to lens epithelial cells in the germinative zone. Agents able to have this effect include products or peroxidation of polyunsaturated fatty acids (abundant in retina rod outer segments and synapses), short wavelength radiation (ultraviolet, x-ray, β, and γ rays), and numerous chemical mutagenic agents such as NMU.

(2) BMC in NMU-injected normal albino rats. Examination of the "control" NMU-injected normal rats, as compared with saline-injected rats, revealed BMC at five months of age in the NMU-injected rats. This histopathological appearance of the cataracts was that of posterior subcapsular cataracts of a severe nature with abnormally large cells of Wendl not only at the posterior pole but encircling the lens; some cells were binucleate. The retina showed advanced generate changes.

(3) Antioxidant diets in RCS rats. Antioxidant diets that prevent the cataracts in pink-eyed RCS dystrophic rats have the effect of retarding retinal degeneration. None of the diets we have tried stops the degeneration, but, when a certain degree of retardation is achieved, the lens is protected. Last year we increased the concentration of vitamin E in the AIN-76 purified diet that already had been supplemented with 0.4 percent β-carotene plus 0.01 percent of BHT (to prevent oxidation of carotene in the cage hopper) as well as 1000 mg/Kg vitamin C and 150 mg/Kg vitamin E. We did not succeed in showing any additional retardation of the retinal degeneration (beyond 55 days), perhaps because apoptosis occurs in this rat strain.

Proposed Course

As pink-eyed, tan-hooded dystrophic breeders become available from the National Institutes of Health (NIH) Foundation Colony, lens

epithelial cell whole mounts will be prepared to compare the numbers of cells in lenses with and without cataract.

Collaborative studies of the mutagen NMU as a cataractogenic agent will continue. Principles learned from chemoprevention in rats with breast and prostate cancer would involve using dual agents that can (1) halt or slow multiplication of lens epithelial cells and (2) cause differentiation into lens fibers.

Collaboration to find the mutant autosomal recessive rdy gene on chromosome 3 of the RCS rat has been investigated. Until now, this study has not been feasible because the rat genome had not been well studied. However, knowledge of the rat genome has been proceeding apace. A molecular biologist in NCRR, VRP, where "fingerprinting" of some of the strains of rats in the Foundation Colonies (including RCS rats) is being pursued, may be interested in this gene, which appears to be involved in RPE phagocytosis.

NEI Research Program

Lens and Cataract—Pathogenesis of Cataract

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Hess HH: Incidence of bilateral mature cataracts in Royal College of Surgeons (RCS) rats and environmental light stress. *Invest Ophthalmol Vis Sci* 35(suppl):2137, 1994.

Laboratory of Immunology

Report of the Chief
Laboratory of Immunology

Robert B. Nussenblatt, M.D.

The Laboratory of Immunology (LI) has finished its eighth year. The sections of the laboratory include: the Immunology and Virology Section headed by Dr. John J. Hooks; the Section on Experimental Immunology headed by Dr. Igal Gery, who is also the deputy head of the Laboratory; the Section of Immunoregulation headed by Dr. Rachel Caspi; the Section on Experimental Immunopathology headed by Dr. Chi-Chao Chan; the Section on Clinical Immunology whose acting head is Dr. Marc de Smet; and the Section on Molecular Biology headed by myself as acting head of an interdisciplinary group.

Section on Clinical Immunology

The Section on Clinical Immunology has continued to focus on major areas of clinical relevance, including new interventional studies. The section has continued its study of patients with acquired immunodeficiency syndrome (AIDS) in collaboration with the National Institute of Allergy and Infectious Diseases (NIAID). A randomized study to evaluate a slow-release implant filled with ganciclovir was tested in AIDS patients with cytomegalovirus (CMV) retinitis. These implants are placed directly into the eye through the pars plana. They have been calibrated to release therapeutically effective doses of ganciclovir over an eight-month period. Recruitment for this study has come to an end, and the results will be tabulated and reported in the near future. This randomized study may yield important information about a new alternative to systemic anti-CMV therapy for patients who cannot tolerate intravenous therapy or those who do not wish to be treated with systemic therapy. The potential for a marked improvement in quality of life in these patients is a serious consideration. Pediatric AIDS patients continue to be seen and evaluated for the incidence of ocular infection. This study is done in conjunction with Dr. Philip Pizzo and the National Cancer Institute (NCI).

The group has been extremely active in the development of new ways in which the ocular immune response can be interfered with. This has included the study of the effect of NPC-15669, an inhibitor of neutrophil recruitment in uveitis. It was found that the injection of this compound into rats will significantly reduce the severity of endotoxin-induced uveitis (EIU), a disease that is mediated mainly by polymorphonuclear cells as well as macrophages. Of great interest as well was that treatment of rats late in the process of experimental autoimmune uveitis (EAU), a disease thought to be mainly mediated by T-cells, could also have a marked inhibition of the evolution of their disease. Of great potential interest as a therapeutic mode in the near future is the effectiveness of humanized anti-interleukin (IL)-2 receptor antibodies in the treatment of autoimmune uveoretinitis in monkeys. This work has been performed in collaboration with Dr. Thomas Waldmann of the NCI as well as Drs. James Raber and

Martin Kriete, from the Veterinary Research and Resources Section, National Eye Institute (NEI). The humanized antitact antibody is an anti-IL-2 receptor antibody originally produced in mice but has been modified by replacing all but its binding region with human immunoglobulin elements. Cynomolgus monkeys induced with EAU were treated with this antibody; the disease was markedly limited in the group treated with the antitact humanized antibody. These findings are in marked contradistinction to the inflammation that was seen in control groups. This work has great significance because of the implications for the potential for human therapeutic studies to occur in the not-too-distant future. From a basic scientific point of view, these studies also implicate the potential role of IL-15 in this ongoing process.

Section on Molecular Biology

This new interdisciplinary group, the Section on Molecular Biology, has been in existence now for more than two years. The attempt is to better understand approaches to gene therapy, whether they be local or systemic in nature. The group has had a long-term interest in focusing on the regulation of the ornithine-δ-aminotransferase (OAT) gene. Work continued in the development of a knockout gene model for this disorder. Additionally, interactions with a variety of individuals, including those from Johns Hopkins University and the State University of New York at Stony Brook, have begun to develop methods by which therapy for gyrate atrophy could be introduced through skin cells. Work by this group has also emphasized studying direct ocular gene transfer using E1A deficient adenovirus constructs. These have been used to transfer genes into isolated retinal pigment epithelial cells (RPE) followed then by transplantation of these RPE cells. A mutant of transforming growth factor beta (TGF-β) has been placed into the adenovirus construct. It appears from early experiments that the RPE cells are capable of sustaining replication of E1A deficient adenovirus.

Section on Immunoregulation

The Section on Immunoregulation has maintained its great interest in the development and study of animal diseases of experimental auto-ocular autoimmune disease. One aspect of the work has been to characterize further murine EAU because the mouse model offers quite important differences from other rodent models of uveitis. The section found a possible correlation between the pathogenicity of autoimmune T-cells and their lymphokine production expression of functional adhesion molecules as well as the expression of some surface antigens in the examination of the Lewis rat model for uveitis. Antigen-specific Lewis rat T-cell lines and sublines have been developed; one is specific for the major pathogenic epitope on the human retinal soluble S-antigen (S-Ag) and three are specific to the major pathogenic epitopes of bovine interphotoreceptor retinoid-binding protein (IRBP). Though these lines have different degrees of uveitogenicity, the four T-cell lines produce roughly equivalent amounts of interferon gamma (IFN-γ) tumor necrosis factor, IL-3, IL-6, and TGF-β.

Of interest, IL-4 cannot be detected. Similarly, there are essentially equal amounts of functional adhesion molecules being expressed; however, the nonpathogenic subline was the poorest responder to antigen stimulation with respect to proliferation in IL-2 production. The nonpathogenic subline will show almost no expression of CD-4. These results would support the contention that class 2 restricted recognition of autoantigens within the neuroretina by uveitogenic T-lymphocytes must occur as an initial step in the induction of experimental uveitis. Therefore, a defect in this step will preclude marked uveitogenicity of these cells. Cytokine genes within the eye in murine experimental uveitis have been studied. T-cells that have been specifically grown in the presence of the retinal protein IRBP or to its peptides can induce uveitis with adoptive transfer. These cell lines show an unrestricted cytokine profile *in vitro*. Looking at messenger ribonucleic acid (mRNA) pro-

duction, a TH-1 type cytokine profile (IL-2, IFN-γ, and tumor necrosis factor alpha [TNFα], was present in the eyes of mice that had EAU induced with the transfer of these cells lines. These results suggest that these lymphokines are important for the induction of uveitis and that a lack of IL-4 mRNA in the eyes of mice that had experimental uveitis would argue that predominately TH-1 type cells were present. Additionally, the group has identified a major pathogenic epitope in the IRBP molecule that is recognized by mice of the H2R haplotype.

Another approach of suppressing ocular autoimmunity was through the induction of oral tolerance. EAU susceptible B-10.A mice were fed IRBP or a control solution. Results indicated that three feedings of 0.2 mL of IRBP every other day before immunization did not protect mice against this form of uveitis, whereas a similar regimen of five doses was in fact protective. However, of interest was supplementing the nonprotective three times regimen with one intraperitoneal administration of recombinant human (rHum) IL-2 resulted in disease suppression that was equal to that of the protective feeding regimen. Analysis of the Peyer's patch cells of fed mice showed a large increase in the production of TGF-β, IL-4, and IL-10 in those animals that were fed IRBP and received IL-2, as compared with those animals that only received IRBP feedings. The group would propose that IL-2 treatment enhances the protection from experimental uveitis by stimulating regulatory cells that ultimately produce cytokines such as TGF-β, IL-4, and IL-10. This interest in immunosuppression oral tolerance has also resulted in an ongoing randomized masked study to look at the effectiveness of oral tolerization. This study continues to be in progress and will evaluate the usefulness of S-Ag oral administration in the induction of tolerance in uveitis patients. It is hoped that this study will be completed in the next year.

Section on Experimental Immunology

The Section on Experimental Immunology has continued its long-term investigation of the pathogenesis of inflammatory eye diseases. In this ongoing interest, they reported the uveitogenicity of recoverin this year. Recombinant recoverin was found to be highly uveitogenic in Lewis rats, inducing a severe experimental uveitis at doses as low as 10 μg per rat. The clinical and histopathologic changes induced by recoverin were very similar to those that one sees with disease induced by the retinal S-Ag. Of great clinical interest is that recoverin has been suggested by many to be the antigen to which antibodies are developed in carcinoma-associated retinopathy syndrome. Work by the group has continued to look at the uveitogenicity and antigenicity of rHumS-Ag in primates. Monkeys of three species have been immunized with recombinant S-Ag molecules.

The ocular changes observed closely resemble those seen in previous studies of monkeys immunized with bovine S-Ag. Additionally, lymphocyte proliferative responses were quite similar to those previously seen. Transgenic mice were also evaluated by this group during the year. Transgenic mice that expressed foreign antigens in their lens were developed by collaborators at the Laboratory of Molecular and Developmental Biology (LMDB), NEI. These foreign antigens were expressed under control of the αA-crystalline promoter. Development of immunotolerance in transgenic mice was examined by measuring their capacity to mount specific immune responses following immunization with a corresponding antigen, which was emulsified in agevin. The main finding showed that the expression of chloramphenicol acetyltransferase (CAT) in the lens had little effect on the

capacity of the transgenic mice to respond against this antigen. Of interest was that in contrast to the findings with CAT, expression of human fibroblasts (HEL) in the lens produced a state of complete tolerance to this antigen. HEL transgenic mice failed to develop any detectable antibody or cellular immune response against this antigen.

The group has also explored the phenomenon of oral tolerance by looking at the oral administration of the bacterial product N-acetyl-D-glucosaminyl-β (1-4)-N-acetyl-L-muranyl-L-alanyl-D-isoglutamine (GMDP). When GMDP was given along with the uveitogenic peptide, it significantly enhanced the level of tolerance induced by the peptide. The role of CD-8 positive lymphocytes in the process that produces oral tolerance was also examined by testing whether mice deficient of these cells were capable of developing oral tolerance. The CD-8 deficient animals used were β-2 m-mice. These mice then have very low expression of major histocompatibility complex (MHC) class 1 molecules and a severe deficiency of CD-8 positive cells. Feeding these mice with ovalbumin produced remarkable levels of immunotolerance that closely resembled those observed in the similarly treated control animals. This finding thus indicated that CD-8 positive cells are not essential for the induction of oral tolerance, at least when induced by the procedure used in this study.

Further work by this group looking at transgenic models has evaluated the effects of IFN-γ on the physiology of the eye and the role of elevated MHC class 2 in the eye. Both transgenic rat and mice strains were generated by microinjection of deoxyribonucleic acid fragment containing a murine αA-crystalline promoter, which was then fused to the coding sequence of murine IFN-γ gene. In both the rat and mouse models, ectopic expression of IFN-γ in the lens affecting the growth of the whole eye resulted in cataract thickening of the anterior lens capsule, rupture of posterior capsule, impairment of the lens fiber formation as well as microphthalmia and microphakia.

The group has an ongoing interest in the role of the T-cell receptor and how it relates to autoimmune intraocular inflammatory disease. The goal is to develop anti-T-cell receptor therapies for the treatment of uveitis. In doing this, athymic as well as euthymic rats were injected with cells from antigen-stimulated T-cell lines specific for a major pathogenic epitope for bovine IRBP. The findings suggest that the time of appearance of cytokine-producing T-cells in the retina is influenced by the immunologic status of the rat, the difference in circulating cytokines in athymic rats might affect parameters such as vascular permeability and could facilitate penetration of T-cells into the eye. Additionally, the strong TH-1 like cytokine profile was detected in R-16 rats, but this was not detected in AK-16 rats. This might suggest that the cytokine profile observed in the retina is influenced by the identity of the pathogenic epitope.

The Clinical Branch, in collaboration with the Experimental Immunology Section, has looked at a variety of immunologic questions as they relate to ocular disease. A continuing interest in cell adhesion molecules has yielded new information. The effective treatment with the monoclonal antibody directed against lymphocyte function-associated antigen (LFA-1) and very late activation antigen (VLA)-4 demonstrates that the anti-LFA1 antibodies significantly inhibited the development of EIU. This was in contrast to the anti-VLA-4 antibody that had no effect on the development of intraocular inflammation. They have also seen that systemic treatment with anti-IL-12 monoclonal antibodies exacerbates the development of EIU. These findings were similar to previous studies with anti-IFN-γ antibody. They have also noted that topical heparin significantly inhibited the development of allergic conjunctivitis in mice.

This group has also been very active in the evaluation of uveitis in patients. The group has been involved in a number of studies designed to improve the treatment of uveitis. They have recently completed a prospective, double-masked randomized study

of diamox for uveitis cystoid macular edema. They have also completed a pilot study examining the efficacy and toxicity of a chemotherapy regimen for therapy of patients with central nervous system (CNS) lymphoma involving the brain or eye. This has been done in collaboration with the Medicine Branch of NCI. A clinical trial is also under way evaluating heparin surface-modified intraocular lens in patients with uveitis.

Section on Experimental Immunopathology

The Section on Experimental Immunopathology has continued to study the immunopathology of various inflammatory cells and ocular resident cells in a variety of experimental models of uveitis. This group has developed a particular expertise in immunohistochemistry and *in situ* hybridization techniques that have provided the whole laboratory with the ability to identify and topographically localize immunocompetent cells.

Additionally, it analyzes the alteration of surface markers on ocular resident cells and the production of cytokines in experimental uveitic models as well as in tissue obtained from patients undergoing surgery. They have demonstrated that higher levels of S-Ag and its mRNA are expressed in nonretinal ocular cells such as the lens, the ciliary body, and the trabecular meshwork of EAU rats. This was particularly so in animals that received long-term steroid therapy. The group has also evaluated several new experimental models for uveitis. Experimental melanin induced uveitis (EMIU) can be induced with immunization using bovine choroidal and RPE melanin protein. EMIU is characterized by a bilateral recurrent iris, scleritis, and choroiditis. The main infiltrating cells in this model are T-cells of CD-4 origin seen in the early stage of the disease and CD-8 positive cells that infiltrate at later stages.

There is an abundant expression of adhesion molecules and MHC class 2 antigens on the ocular resident cells one to two days before ocular inflammation is noted. Of interest in this disorder is the fact that recurrences occur one month after the first attack becomes quiescent. The group has continued its interest in evaluating an animal model for acquired ocular toxoplasmosis. This is done by the infection of a virulent strain of *Toxoplasma gondii* (ME49) into mice. Focal ocular inflammation as well as RPE involvement are seen about two weeks after the infection. About one month after infection, ocular inflammation becomes stable and only occasional cysts can be seen. The group evaluated 12 cases of intraocular lymphoma diagnosed at the NEI between 1984 and 1992. These were all non-Hodgkin's large B-cell lymphomas of the CNS. The prompt appropriate handling of specimens and the review by an experienced cytopathologist have been shown to be exceptionally critical to the diagnosis of intraocular lymphoma. They have also evaluated three affected members of a Chinese-American family with Bietti's crystalline retinopathy. Crystalline lysosomal materials are observed in lymphocytes and skin fibroblasts of these patients.

Section on Immunology and Virology

The Section on Immunology and Virology has continued to emphasize its interest in the study of the RPE cells. This section has developed a new method using RPE choroidal explants to initiate cell growth. By monitoring the clusters of cells growing around the explants, they were able to select purely epithelial cells and discard the nonepithelial cells at the primary culture stage. Using this technique, they have established primary cell lines of human RPE from cells of elderly patients. The cell culture origin was confirmed by immunochemical staining for cytokeratin with monoclonal antibodies. Human RPE cultures secrete significant quantities of IL-6 and intercellular adhesion molecule 1 (ICAM-1) but no IL-1 in response to stimulation by inflammatory mediators.

These observations are important in understanding posterior uveitis that may be caused by infections or an autoimmune process. Lymphocytes and macrophages infiltrate into the retina and secrete cytokines such as IL-1, TNFα, IFN-γ, and IL-2 that would initiate immune reactions. In response to these cytokines, the retinal resident cells could locally produce IL-6 as well as ICAM-1 to amplify the immunopathologic process. The group has continued its interest in the field of ocular toxoplasmosis. They were able to demonstrate in this model that 100 percent of mice develop cysts in the brain, but retinal cysts could be found late in the course of the disease. This section has also developed polymerase chain reaction techniques for the detection of a variety of viruses in the eye. This ability will be applied in the future for the evaluation of intraocular samples for the presence of a variety of viruses that are thought to be pathogenic in the eye.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00280-03 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transgenic Rat and Mouse Models for the Study of Intraocular Effects of IFN-γ and Autoimmunity

PRINCIPAL INVESTIGATOR *(List othar professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Charles E. Egwuagu	Ph.D., M.P.H.	Senior Scientist	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunology	LI, NEI
	Ana B. Chepelinsky	Ph.D.	Head, Section on Regulation of Gene Expression	LMDB, NEI
	Jorge Sztein	D.V.M., Ph.D.	Visiting Associate	LI, NEI
	Rashid Mahdi	B.S.	Biologist	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Experimental Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To study the possible role of interferon (IFN)-γ in ocular pathogenesis and, specifically, the linkage between its induction of aberrant major histocompatibility complex (MHC) class II expression and predisposition to ocular autoimmune diseases, we generated transgenic mice with constitutive expression of IFN-γ in the eye. Although the mouse has, up to this point proven to be a useful model, the rat is the preferred strain for the study of experimental autoimmune uveoretinitis (EAU). EAU is an animal disease that shares essential features with several human uveitic diseases such as sympathetic ophthalmia, birdshot retinochoriodopathy, Behçet's disease, and Vogt-Koyanagi-Harada (VKH) syndrome. Consequently, we have generated a IFN-γ transgenic Sprague Dawley rat strain. In FY 1993-1994 we focused on characterizing both the rat and mouse IFN-γ transgenic models with the goal of establishing a comprehensive and complementary transgenic animal system that would be useful for studying the *in vivo* effects of IFN-γ in the eye.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The objectives of this project include the study of the possible role of interferon gamma (IFN-γ) in the eye. Aberrant expression of the major histocompatibility complex (MHC) class II molecules is an early event in a number of human autoimmune diseases, and IFN-γ induces high levels of MHC class II protein biosynthesis. Therefore, we generated transgenic animals with selective secretion of IFN-γ in their eye tissues. These transgenic mice and rats are ideally suited for studying the effects of IFN-γ on the physiology of the eye and the role of elevated MHC class II predisposition to intraocular autoimmune diseases.

Methods

Transgenic rat and mouse strains were generated by microinjection of a deoxyribonucleic acid fragment containing the murine αA-crystallin promoter (αACry) fused to the coding sequence of murine IFN-γ gene. Polymerase chain reaction (PCR) and reversed transcription-PCR (RT/PCR) were used to screen for the presence of the transgene and conduct messenger ribonucleic acid (mRNA) analyses, respectively. Methacrylate-embedded eye sections were analyzed for morphology and cryosections for immunoperoxidase antibody staining.

Major Findings

In both rat and mouse models, ectopic expression of IFN-γ in the lens affected the growth of the whole eye, resulting in cataract, thickening of anterior lens capsule, rupture of posterior capsule, impairment of lens fiber formation, microphthalmia, and microphakia. Additional effects in the mouse include blepharophimosis, arrest of retinal differentiation, serous retinal detachment with presence of macrophages in the subretinal space, persistent hyperplastic primary vitreous, and corneal vascularization. Unlike the mouse, the rat anterior chamber is well formed, and the retina is intact with focal retinal serous detachment. MHC class II mRNA levels were significantly increased in the transgenic mouse eyes, and MHC class II proteins were expressed in their corneas, irises, ciliary bodies, choroids, lens, and retinal pigment epthelia. At the molecular level, the pattern of lens gene expression was perturbed, and expression of gene coding for adhesion molecules and IFN-γ-inducible transcription factor was up-regulated in transgenic eyes.

Significance to Biomedical Research and the Program of the Institute

The αACry/IFN-γ transgenic rat is the first transgenic rat strain generated for vision research. Constitutive expression of IFN-γ, and its induction of MHC class II molecules in the eye, provides a useful model to address: (1) the linkage between aberrant MHC class II expression and predisposition to autoimmunity, (2) the role of IFN-γ in the treatment of inflammatory eye diseases and in ACAID, and (3) understanding cytokine signaling during embryonic eye development. The rat and mouse models complement each other for elucidation of the *in vivo* effects of IFN-γ in the eye.

Proposed Course

We intend to continue studying the molecular basis of IFN-γ actions in the eye with particular emphasis on the rat. A major focus will be to establish primary and long-term cultures of IFN-γ-expressing epithelial lens cells as these cell lines would be valuable in studies aimed at understanding the mechanism of transcriptional activation in the αACry-IFN-γ animals.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Egwuagu CE, Sztein J, Reid W, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Gamma interferon expression disrupts lens

and retinal differentiation in transgenic mice. *Dev Biol,* in press.

Egwuagu CE, Sztein J, Reid W, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Transgenic rat and mouse models for studying the role of gamma interferon and MHC Class II in intraocular diseases and autoimmunity, in Nussenblatt RB, Gery I (eds), *Sixth International Symposium of the Immunology and Immunopathology of the Eye.* Amsterdam, Netherlands, Elsevier Press, in press.

Egwuagu CE, Sztein J, Reid W, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Ectopic expression of gamma interferon in the eyes of transgenic mice induces ocular pathology and MHC class II gene expression. *Invest Opthalmol Vis Sci* 35:332-341, 1994.

Egwuagu CE, Sztein J, Reid W, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Transgenic rat and mouse models for the study of intraocular effects of IFN-γ and autoimmunity. *Invest Opthalmol Vis Sci* 35/4 (suppl):3391, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00262-05 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of T Lymphocytes and Cytokines Involved in Experimental Autoimmune Uveoretinitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Charles E. Egwuagu	Ph.D., M.P.H.	Senior Research Scientist	LI, NEI
Others:	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Rachel Caspi	Ph.D.	Visiting Associate	LI, NEI
	Rashid Mahdi	B.S.	Biologist	LI, NEI
	Alexanda T. Kozhich	Ph.D.	Visiting Fellow	LI, NEI
	Phyllis B. Silver	B.S.	Biologist	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Experimental Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Experimental autoimmune uveoretinitis (EAU) is a T-cell-mediated autoimmune disease that serves as a model of human intra-ocular inflammatory diseases (uveitis). It is initiated in susceptible animals by immunization with retinal antigens such as interphotoreceptor retinoid-binding protein (IRBP) or S-antigen (S-Ag) or by adoptive transfer of activated S-Ag- or IRBP-specific uveitogenic T lymphocytes. We had previously demonstrated that Vβ8-expressing T cells accumulate in the retina during EAU. In FY 1993-1994, we sought to define the T-cell subsets and cytokines present in the retina of athymic and euthymic Lewis rats after adoptive transfer of uveitogenic or nonuveitogenic T lymphocytes. Our results indicate that (1) the temporal appearance of cytokine-producing T cells in the retina is influenced by the immunological status of the rat and might affect parameters such as vascular permeability that influence the penetration of lymphocytes into the eye. (2) Cytokine messenger ribonucleic acid (mRNA) transcripts were detected in the retinas of animals immunized with uveitogenic T lymphocytes, as well as in the retinas of rats injected with Con A-specific T cells. However, rats injected with Con A-specific T cells neither developed EAU nor was there detection of $V\beta8^+$ T cells in their retinas. (3) Detection of interferon (IFN)-γ transcripts was temporally correlated with the appearance of $V\beta8^+$ T cells in the retina and the onset of disease. Taken together, our data suggest that infiltration of the retina by activated T-cells is not sufficient for disease induction; ocular-antigen specific Th1-like $V\beta8^+$ lymphocytes appear to be necessary for EAU induction.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

This project is aimed at determining the clonality of the T lymphocytes that mediate intraocular autoimmune diseases. Identification of the pathogenic T-cell subset in experimental autoimmune uveoretinitis (EAU) is relevant to our goal of developing anti-T-cell receptor (TCR) therapies for the treatment of uveitis. Our effort during fiscal year 1993-1994 focused on analyses of T cells present at the autoimmune site and the lymphokines that they produce.

Methods

Athymic and euthymic rats were injected with cells from an antigen (Ag)-stimulated T-cell line specific to the major pathogenic epitope of bovine interphotoreceptor retinoid-binding protein (IRBP) (peptide R16: amino acids 1177-1191; R16 rats) or with concanavalin A (ConA)-stimulated splenocytes (ConA rats). In a separate experiment, euthymic rats were injected with an Ag-stimulated T-cell line specific to the pathogenic epitope of rat IRBP (peptide AK16: amino acids 273-283; AK16 rats) or with purified proton derivative (PPD)-stimulated primed lymph node cells (PPD rats). Retinas were sampled every 12 hours after uveitogenic challenge and reversed transcription polymerase chain reaction (RT/PCR) was used to analyze messenger ribonucleic acid (mRNA) levels for TCR Vβ8, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin (IL)-2, IL-6, CD-4, and CD-8.

Major Findings

(1) Analyses of cytokine mRNAs in retinas of athymic and euthymic ConA and R16 rats showed that in athymic rats IFN-γ, TNF-α, IL-2, IL-6, CD-4, and CD-8 mRNAs were detected after 12 hours, whereas in the euthymic rats they were only detected after 24-84 hours. Retinas of ConA rats showed essentially the same cytokine profile as retinas of R16 rats. Cytokine mRNAs were not detected in retinas of animals that did not receive cells.

(2) Analyses of cytokine mRNAs in euthymic AK16 and PPD rats showed that TNFα, IL-6, CD-4, and CD-8 mRNAs were detected after 24 hours, but IFN-γ and IL-2 transcripts were not detectable even after 120 hours. In contrast to ConA rats, cytokine mRNA expression was not detected in retinas of PPD rats. (3) In all experiments, the appearance of Vβ8$^+$ T cells in the retina coincided with the temporal expression of IFN-γ in the retinas of rats with experimental autoimmune uveitis (EAU). A similar correlation was not observed for any of the other cytokines.

Significance to Biomedical Research and the Program of the Institute

(1) The time of appearance of cytokine-producing T cells in the retina appears to be influenced by the immunological status of the rat. Differences in levels of circulating cytokines in athymic rats might affect parameters such as vascular permeability and could facilitate penetration of T cells into the eye.

(2) A Th-1-like cytokine profile was detected in R16 rats, whereas it was not detected in AK16 rats. This might suggest that the cytokine profile observed in the retina is influenced by the identity of the pathogenic epitope.

Proposed Course

Analyses of uveitogenic T-cell clonotypes and the lymphokines they produce during EAU will be continued to identify the relevant autoaggressive T cells involved. Our study will be expanded to include analyses of specimens obtained from patients with ocular sarcoidosis and anterior and posterior uveitis.

NEI Research Program

Retinal Diseases—Inflammatory Disorders

Publications

Egwuagu CE, Bahmanyar S, Mahdi R, Nussenblatt RB, Gery I, Caspi R: Predominant usage of Vβ8.3 T cell receptor in a T cell line that induces experimental autoimmune uveoretinitis. *Clin Immunol & Immunopathol* 65:152, 1992.

Egwuagu CE, Caspi R, Mahdi R, Gery I, Nussenblatt BR: Evidence for selective accumulation of Vβ8+ T lymphocytes in experimental autoimmune uveoretinitis induced by two different retinal antigens *J Immunol* 151:1627, 1993.

Kozhich AT, Kawano Y, Egwuagu CE, Caspi RR, Maturi RK, Berzofsky JA, Gery I: A pathogenic autoimmune process targeted at a surrogate epitope. *J Exp Med,* in press

Mahdi RM, Caspi RR, Kozhich AT, Kozhich OA, Silver PB, Nussenblatt RB, Egwuagu CE: Cytokine mRNA expression following adoptive transfer of uveitogenic T cells into athymic and euthymic Lewis rats. *Invest Opthalmol Vis Sci* 35/4 (suppl):1432, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00069-17 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immune Responses to Ocular Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
Others:	Alexander Kozhich	Ph.D.	Visiting Fellow	LI, NEI
	Eddy Anglade	M.D.	Senior Staff Fellow	LI, NEI
	Scott M. Whitcup	M.D.	Associate Clinical Director	CB, NEI
	Chi-Chao Chan	M.D.	Head, Section of Immunopathology	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	LI, NEI
	Barbara Vistica	B.A.	Microbiologist	LI, NEI
	Nathan Felix	B.A.	Special Volunteer	LI, NEI
	James Lai	B.A.	Guest Researcher	LI, NEI
	Eric Wawrousek	Ph.D.	Head, Section on Transgenic Animals and Genome Manipulation	LMDB, NEI
	Christina M. Sax	Ph.D.	Senior Staff Fellow	LMDB, NEI

COOPERATING UNITS *(if any)*

Biotechnology Unit, Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases (Joseph Shiloach, Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Experimental Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892-1858

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.8	2.4	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Targeted at learning about inflammatory eye diseases grouped under the term "uveitis," this project continued to focus mainly on learning about ocular antigens capable of inducing experimental autoimmune uveoretinitis (EAU), an animal model for uveitis in humans, and procedures that modulate this disease. The major achievements of this project in FY 1994 include: (1) We discovered that recoverin, a retinal calcium-binding protein, is highly uveitogenic in rats, producing severe inflammatory changes in eyes of rats of all four strains tested. In addition to identifying a new uveitogenic retinal molecule, this observation provides evidence to support the assumption that recoverin is the target for an autoimmune process that causes a condition termed cancer-associated retinopathy (CAR). (2) Recombinant human S-antigen, which has become available by recombinant deoxyribonucleic acid (DNA) technologies, was found to be uveitogenic in primates, inducing severe inflammatory ocular changes that closely resemble those induced by bovine S-antigen. Lymphocytes from the immunized monkeys were used to identify the peptides that are selected as the immunodominant determinants, i.e., the ones that are the target for the immune response in animals immunized with the whole protein. Monkeys of different species responded against different peptides, whereas four monkeys of the same species (cynomolgus) remarkably responded against the same selected peptides. (3) Blood lymphocytes collected at different time points from a human donor exhibited consistency in their responding strongly to the same selected peptides of human S-antigen. Yet, marked changes were observed in the capacity of individual dominant peptides to stimulate lymphocytes collected at different time points. (4) To examine the effect of sequestration on the immunogenicity of lens proteins, transgenic (TG) mice were developed in which foreign antigens are selectively expressed in the lens. Two different types of response were observed: mice expressing chloramphenicol aminotransferase (CAT) responded to this antigen similarly to their wildtype controls, while TG mice expressing hen egg lysozyme (HEL) failed to respond against this antigen, due to a state of complete immunotolerance. (5) Oral tolerance, a procedure used to inhibit pathogenic autoimmune processes, was found to be enhanced by treating the fed animals with certain bacterial products, with the best effect achieved with glucosaminyl muramyl dipeptide (GMDP). (6) The role of CD8 lymphocytes in the process of oral tolerance induction was examined by testing the capacity of mice deficient in these cells to develop oral tolerance. CD8 deficient mice resembled their controls in developing tolerance, thus showing that CD8 cells are not always essential for induction of oral tolerance.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

Studies conducted during fiscal year (FY) 1994 were aimed at the following: (1) to examine the uveitogenicity of recoverin, a calcium-binding protein specific to the retina, that was reported to be the target for autoantibodies detected in the majority of cases with cancer-associated retinopathy (CAR); (2) to test the uveitogenicity of recombinant human S-antigen (rHumS-Ag) in primates and to identify the peptide determinants that are selected as the immunodominant epitopes by the immune system of the immunized monkeys; (3) to monitor at different timepoints the changes that may occur among the subpopulations of human blood lymphocytes that respond against different epitopes on HumS-Ag; (4) to investigate the level of sequestration of proteins within the lens by testing the development of immunotolerance toward foreign antigens expressed in the lens of transgenic mice (expression of foreign proteins in other organs usually produces tolerance); (5) to test the capacity of bacterial products to enhance oral tolerance, a procedure that is extensively used to inhibit pathogenic autoimmune processes, including uveitis in man. In the system used here, feeding with S-Ag is used to inhibit the development of experimental autoimmune uveoretinitis (EAU) in rats; and (6) to investigate the role of CD-8 lymphocytes in the process that brings about oral tolerance by testing the capacity of CD-8 deficient mice to develop oral tolerance.

Methods

Recombinant recoverin was kindly provided by Dr. L. Stryer, from Stanford University; rHumS-Ag was prepared by Dr. Joseph Shiloach, from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), as described in our *Annual Report*, FY 1993; peptides were synthesized and purified by Applied Biosystem, Foster City, California. Monkeys of different species (rhesus, artcoides, and cynomolgus) were provided by the National Institutes of Health (NIH) animal facility, and R2 m -/- mice (CD-8 deficient) were provided by the NCI Twinbrook Facility. Blood samples were collected at different timepoints from a single donor, and the mononuclear leukocytes were separated on Isolymph gradients. Published conventional methods were used to immunize animals and measure their immune response as well as development of EAU. Adoptive transfer of EAU was carried out as described by Mochizuki et al. (*Invest Ophthalmol Vis Sci* 26:1, 1985). Levels of chloramphenicol acetyltransferase (CAT) were measured by a biphase CAT assay, and those of human fibroblasts (HEL) were determined by the particle concentration fluorescence immunoassay.

Major Findings

Uveitogenicity of recoverin. Recombinant recoverin was found to be highly uveitogenic in Lewis rats, inducing severe EAU at doses as low as 10 μg per rat. The clinical and histopathological changes induced by recoverin closely resemble those elicited in rats by S-Ag, reaching in many cases the maximum grade of severity of 4+. Recoverin is also found in the pineal gland, and most rats with EAU also developed pineal inflammation. In addition to Lewis rats, recoverin was found to induce EAU in three other rat strains—BN, WF, and ACI. Similarly to observations with other uveitogenic antigens, however, the severity of changes in these strains was lower than in Lewis rats. EAU induced by recoverin was found to be cell-mediated; it is readily and adoptively transferred to naive recipients by lymph node or spleen cells from immunized donors. Moreover, antibodies to recoverin seem to play a minor role, if any, in the pathogenic process because recipients developed disease even in the absence of antibodies, as seen in rats injected with recoverin-sensitized spleen cells that were stimulated in culture with Concanavalin A.

Uveitogenicity and antigenicity of rHum S-A8 in primates. Monkeys of three different species (artcoides, rhesus, cynomolgus) developed uveitis when immunized with rHumS-Ag.

The ocular changes closely resembled those observed in previously published studies in eyes of monkeys immunized with bovine S-Ag (Nussenblatt RB, et al., *Arch Ophthalmol* 99:1090, 1981). Peripheral blood lymphocytes from the immunized monkeys responded well against the rHumS-Ag molecule as well as against bovine S-Ag.

The monkey lymphocytes were also tested for their proliferative response against 40 synthetic overlapping peptides that span the entire sequence of HumS-Ag. Lymphocytes from monkeys of the three tested species recognized and responded well against different peptides; a finding that indicates that different determinants of HumS-Ag serve as the "immunodominant epitopes" for the immune system of monkeys of different genetic makeups. On the other hand, all four cynomolgus monkeys remarkably recognized the same immunodominant regions of HumS-Ag at sequences 21-61, 71-90, 121-140, 171-200, and 281-310.

Response of human lymphocytes against HumS-A8 peptide determinants: Specificity pattern is conserved. Peripheral blood lymphocytes collected from a single donor at different timepoints were used to examine the possible changes in the repertoire of responsiveness toward HumS-Ag and its 40 overlapping peptides. Six blood samples, collected at timepoints spanning over six months, were tested in the present study. Significant responses were found at all timepoints against four regions, localized at sequences 71-90, 121-140, 171-200, and 341-360. Marked variations were noted, however, among the six blood samples in the level of response to each of these four peptides, thus producing differences in the hierarchy of their stimulating capacity.

Immune responses in transgenic mice expressing foreign antigens in their lens. Transgenic mice that express foreign antigens in their lens were developed by collaborators at the LMDB, NEI. Mice expressing CAT were developed by Dr. Chris Sax, but mouse lines expressing HEL were established by Dr. Eric Wawrousek. Both foreign antigens were expressed under the control of the αA-crystallin promoter. Sensitive immunological tests (see Methods section) showed high concentrations of CAT or HEL in the lens, but neither antigen could be detected in any other organ or in the blood of the transgenic mice. Development of immunotolerance in the transgenic mice was examined by measuring their capacity to mount specific immune responses following immunization with the corresponding antigen, emulsified in complete Freund's adjuvant. The main findings include: (1) Expression of CAT in the lens had little effect on the capacity of the transgenic mice to respond against this antigen. Transgenic mice immunized with CAT produced antibodies to CAT with levels similar to those of their wild-type controls. The transgenic mice also developed cellular immune response to CAT, albeit with levels slightly lower than those monitored in the wild-type controls. (2) In contrast to the findings with CAT, expression of HEL in the lens produced a state of complete tolerance to this antigen; HEL transgenic mice failed to develop any detectable antibody or cellular immunity against HEL following immunization with this antigen.

Enhancement of oral tolerance by bacterial products. Oral administration of the bacterial product N-acetyl-D-glucosaminyl-β (1-4)-N-acetyl-L-muramyl-L-alanyl-D-isoglutamine (GMDP) along with a uveitogenic peptide significantly enhanced the level of tolerance induced by the peptide. The study was carried out in rats in which EAU is induced by peptide 1181-1191 of bovine interphotoreceptor retinoid-binding protein (IRBP). Feeding with peptide 1181-1191 reduces the disease development, and the level of inhibition was further enhanced by cofeeding with GMDP. A slight enhancing effect was also induced by bacterial lipopolysaccharide (LPS), but *Salmonella typhimurium* mitogen (STM) and the des-(N-acetyl-D-glucosaminyl) analog of GMDP had no detectable effect in this system. The capacity of GMDP to enhance oral tolerance was further demonstrated by the finding that the cellular immunity to peptide 1181-1191 was inhibited remarkably more in rats fed

with the combination of this peptide and GMDP than in those fed with the peptide alone.

Induction of oral tolerance in CD-8 cell-deficient mice. The role of CD-8 lymphocytes in the process that produces oral tolerance was examined by testing whether mice deficient of these cells are capable of developing oral tolerance. The CD-8-deficient animals used here were β2m -/- mice, *i.e.*, animals in which the disruption of the β2-microglobulin gene causes very low expression of MHC class I molecules and severe deficiency of CD-8⁺ cells. Feeding these mice with ovalbumin (three or five times, 1 mg per mouse) produced remarkable levels of immunotolerance that closely resembled those observed in the similarly treated control animals. This finding indicates that the CD-8 cells are not essential for the induction of oral tolerance, at least when induced by the procedure used in this study.

Significance to Biomedical Research and the Program of the Institute

(1) The finding that recoverin is highly uveitogenic underscores the unique characteristic of the retina, *i.e.*, its content of multiple molecules with the capacity of initiating pathogenic autoimmune processes; previous studies have identified four other uveitogenic proteins in the retina. In addition, the capacity of recoverin to initiate a pathogenic autoimmune process supports the notion that this protein is the target for the putative autoimmune process that brings about the retinal damage in CAR.

(2) The present study is the first to show that HumS-Ag is highly uveitogenic in primates. Moreover, this observation supports the notion that autologous S-Ag plays a major role in the immunopathogenic process of uveitis in man. This study also provides, for the first time, information concerning the peptide determinants of HumS-Ag that are immunodominant in monkeys in which immunization with HumS-Ag produced uveitis. As expected, monkeys of different species varied in their selection of the dominant peptides, but the similarity among the four cynomolgus monkeys was quite surprising because these animals are outbred. The latter observation may suggest that uveitic patients with partial identity of histocompatibility antigens may also exhibit similarity in their selection of immunodominant epitopes. (Such an observation was made with multiple sclerosis patients).

(3) The study of responsiveness to HumS-Ag peptides of a human donor at different timepoints has provided information concerning the fluctuations among lymphocyte clones with specificity toward autologous peptides. The finding that responses to the same few epitopes were observed in all blood samples thus indicates that only small and quantitative fluctuations occur among the clones of lymphocytes that recognize the dominant epitope of an autologous antigen.

(4) The experiments with the transgenic mice have yielded new information on the development of immunotolerance against antigens expressed inside the encapsulated lens. The findings so far, of two patterns of response against CAT or HEL suggest that lens proteins can be treated in different ways by the immune system.

(5) The observation that the efficacy of oral tolerance can be enhanced by cotreating the animals with GMDP provides a new strategy for the inhibition of pathogenic immunemediated processes such as uveitis. The potential to enhance oral tolerance is of great importance because the feeding procedure usually produces only partial inhibition of autoimmune diseases.

(6) The finding that CD-8-deficient mice develop oral tolerance shows that this subpopulation of lymphocytes is not essential for the induction of oral tolerance in the system used in the present study. CD-8 cells were shown in other studies to play a major role in the induction of oral tolerance (Weiner H, et al., *Annu Rev Immunol* 12:809, 1994), and thus, our data provide direct evidence to the notion that

at least two different mechanisms participate in this process.

Proposed Course

Our future efforts will focus on the following issues: (1) Other retinal proteins, mainly those related to recoverin, will be tested for uveitogenicity; (2) the response to HumS-Ag of primates and human subjects will be further analyzed, mainly by attempts to establish and analyze cell lines with specificity toward this molecule and its peptide determinants; (3) the development of tolerance against foreign antigens expressed in the lens will be further analyzed, mainly with regard to the mechanisms that bring about tolerance and the difference between the responses to CAT and HEL; and (4) more effort will be focused on studies aimed at enhancing oral tolerance, the mechanisms involved in this phenomenon, and its usage for suppression of pathogenic autoimmune processes.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Chan C-C, Hikita N, Dastgheib K, Whitcup SM, Gery I, Nussenblatt RB: Experimental melanin-protein induced uveitis in the Lewis rat: Immunopathological processes. *Ophthalmology* 101:1275-1280, 1994.

Gery I, Chanaud NP III, Anglade E: Recoverin is highly uveitogenic in Lewis rats. *Invest Ophthalmol Vis Sci* 35:3342-3345, 1994.

Gery I, Streilein JW: Autoimmunity in the eye and its regulation. *Curr Opinion Immunol*, in press.

Kasner L, Chan C-C, Whitcup SM, Gery I: The paradoxical effect of tumor necrosis factor alpha (TNF-α) in endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci* 34:2911-2917, 1993.

Kozhich AT, Kawano YI, Egwuagu GE, Gaspi RR, Maturi RK, Berzofsky JA, Gery I: A pathogenic autoimmune process targeted at a surrogate epitope. *J Exp Med* 180:133-140, 1994.

Sasamoto Y, Kawano YI, Wiggert B, Chader GJ, Gery I: Induction of unresponsiveness in adult rats by immunodominant and nondominant peptides. *Cell Immunol* 152:286-292, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00298-01 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Inhibition of EAU in Monkey With Humanized Anti IL-2 Receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	François G. Roberge	M.D.	Visiting Scientist	LI, NEI
Others:	Yan Guex-Crosier	M.D.	Special Volunteer	LI, NEI
	Igal Gery	Ph.D.	Deputy Laboratory Chief	LI, NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	LI, NEI

COOPERATING UNITS *(if any)*

VRRS, NEI (James Raber, Ph.D.); VRRS, NEI (Martin Kriete, Ph.D.); NCI (Thomas Waldmann, M.D.); Hoffmann LaRoche, Nutley, NJ (John Hakimi)

LAB/BRANCH

Laboratory of Immunology

SECTION

Clinical Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Experimental autoimmune uveoretinitis (EAU) was induced in Cynomolgus monkey by immunization with recombinant human retinal S-antigen. At the onset of ocular disease, animals were treated for 28 days with intravenous injection of humanized anti-Tac, an anti-interleukin (IL)-2 receptor antibody originally produced in mouse but modified by replacing all but its binding region with human immunoglobulin elements. Controls were treated with vehicle alone. The animals were examined twice a week during the treatment period. The progression of the disease was markedly limited in the group treated with anti-Tac-H, while the severity of the inflammation continued to increase in the control group. The *in vivo* results were correlated with a significant inhibition of monkey lymphocyte proliferation stimulated by IL-2 when anti-Tac-H was added to the culture medium.

Project Description

Clinical Protocol Number

93255

Objectives

General Goal of the Study. Evaluate the effectiveness of humanized anti-interleukin (IL)-2 receptor antibodies for the therapy of autoimmune diseases.

Specific Objectives

(1) Evaluate the effect of two humanized anti-IL-2 receptor antibodies, anti-Tac-H, and Mik-b1-H, respectively directed at the α and β chain of the IL-2R, in the treatment of active uveoretinitis in monkey.

(2) Evaluate the possibility of a beneficial therapeutic effect in using anti-Tac-H and Mik-b1-H in combination or in the form of recombinant hybrid molecules.

(3) Correlate the therapeutic response with *in vitro* study of inhibition of proliferation of monkey lymphocytes by anti-Tac-H and Mik-b1-H.

(4) Measure the response of treated monkeys against the antibodies used for treatment.

Methods

Animal Immunization and Examination. Cynomolgus monkeys, between 2.0 and 3.0 kg, are immunized with recombinant human retinal S-antigen at 40 μg/kg emulsified in Hunter's TiterMax adjuvant by subcutaneous injection at the nape of the neck.

Twice a week, starting 14 days after immunization, the animals are sedated with ketamine, the pupils dilated with topical atropine, and the eyes examined by indirect ophthalmoscopy for signs of uveoretinal inflammation.

Treatment Protocol. The animals are randomized in two groups of five individuals. Treatment consists of intravenous injection of anti-Tac-H or Mik-b1-H at 2.0 mg/kg, or control vehicle. The treatment is instituted on the day of presentation of the first sign of experimental autoimmune uveitis. Antibody injections are done following a schedule of alternating three- and four-day periods, for a total of eight injections, covering a total treatment period of 28 days. In addition, the pupils are kept dilated with the application of atropine ophthalmic ointment 0.5 percent.

Tests Performed

Preimmunization

- Blood drawn for complete blood count (CBC) and serum for baseline measurements.

At initiation of therapy

- Funduscopy and fundus photography.
- Fundus fluorescein angiogram.
- Measurement of intraocular pressure with a Tonopen tonometer.
- Blood drawn for CBC, serum creatinine, sIL-2R, and serum for baseline measurements.

Once a week

- Fundus photography.
- Measurement of intraocular pressure with a Tonopen tonometer.
- Blood collection for anti-idiotypic antibody and sIL-2R, anti-S-Ag Ab, and level of anti-Tac-H or Mik-b1-H.

At termination of experiment

- Funduscopy and fundus photography.
- Fundus fluorescein angiogram.
- Measurement of intraocular pressure with a Tonopen tonometer.
- Blood drawn for CBC, serum creatinine, anti-idiotypic antibody, sIL-2R, anti-S-Ag Ab, level of anti-Tac-H or Mik-b1-H.
- Eyes are collected for histopathological examination.

Major Findings

On average, the ocular inflammation was stabilized in the animals treated with anti-Tac-H. There was a marked progression of the disease in the control group. Given the caveat of the small sample number, the statistical analysis of the present data by the Mann-Whitney test on the averaged variation per animal showed significance at a value of $p < 0.01$. We also observed a marked decrease of the intraocular pressure (-50 percent) in the inflamed eyes. There was no significant difference between the two treatment groups.

We have also tested the activity of various anti-IL-2 receptor antibodies in the inhibition of IL-2 driven proliferation of monkey peripheral blood lymphocytes. The results confirmed the inhibitory effect of anti-Tac-H and a hybrid of anti-Tac-H and Mik-b1-H that had been observed with human lymphocytes. Mik-b1-H alone was not inhibitory but produced an increased inhibition when used in combination with anti-Tac-H. We were also surprised to find that the antibody 7G7/B6, which does not block the IL-2 signal on human lymphocytes, was a strong inhibitor of monkey lymphocyte proliferation.

In conclusion, the results of this first experiment indicate that the humanized anti-Tac antibody could be useful in the therapy of some autoimmune diseases. We are now conducting a similar experiment to evaluate Mik-b1-H. In light of the results of that experiment, we should decide in what direction to develop the work. Already we feel that the next experiment should include a group to confirm the results obtained with anti-Tac-H. We are also trying to obtain IL-15 to pursue the *in vitro* evaluation of the activity of the monoclonal antibodies on the monkey lymphocytes.

Significance to Biomedical Research and the Program of the Institute

The effectiveness of humanized anti-Tac in monkey suggests that this therapeutic approach could be useful in the treatment of autoimmune diseases in human. Because the molecule is mostly of human origin, it appears that it would be better tolerated in patients, thereby avoiding the production of neutralizing antibodies.

Proposed Course

We will conduct a similar experiment to evaluate Mik-b1-H. In light of the results of that experiment, we will decide in what direction to develop the work, possibly using a combination of antiTac-H with Mik-b1-H. A forthcoming experiment should include a group to confirm the results obtained with anti-Tac-H. We are also trying to obtain IL-15 to pursue the *in vitro* evaluation of the activity of the monoclonal antibodies on the monkey lymphocytes.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00300-01 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of the Effect of NPC 15669, an Inhibitor of Neutrophil Recruitment in Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	François G. Roberge	M.D.	Visiting Scientist	LI, NEI
Others:	Margaret Cheung	M.D., Ph.D.	Senior Staff Fellow	LI, NEI
	Kourosh Dastgheib	M.D.	Visiting Fellow	LI, NEI
	Seiji Hayashi	M.D.	Volunteer Fellow	LI, NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Clinical Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Polymorphonuclear neutrophils (PMN) participate in the inflammatory infiltrate of uveitis. At present, the importance of the role played by these cells is largely unknown. We have studied the influence that an inhibitor of PMN recruitment could have on two types of uveitis: (1) endotoxin-induced uveitis (EIU), a disease mediated mainly by PMN and macrophages, and (2) experimental autoimmune uveoretinitis (EAU), a T-cell-mediated disease in which the early infiltrate is composed mainly of PMN. We have found that injection of NPC 15669 in rats significantly reduced the severity of EIU. More surprisingly, treatment of rats late in the process of EAU induction also inhibited the evolution of this disease.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

(1) Evaluate the effect of NPC 15669 in the inhibition of protein exudation and cellular infiltration in the anterior chamber of the eye after subcutaneous injection of endotoxin.

(2) Evaluate the effect that the inhibition of polymorphonuclear neutrophils recruitment could have on the evolution of experimental autoimmune uveitis (EAU) in the rat.

Methods

Uveitis was induced in Lewis rats with a subcutaneous injection of lipopolysaccharide (LPS). Treatment with NPC 15669 started three hours after injection of endotoxin. NPC at doses of 12, six, or three mg/kg or vehicle alone were given by intraperitoneal (i.p.) injections repeated six times at two-hour intervals. After 24 hours, one eye was collected for histology, and the aqueous humor was aspirated from the other eye for the measurement of protein and cells in the exudate. For EAU, Lewis rats were immunized with S-antigen (S-Ag) in Hunter's adjuvant. NPC was given three times a day at 30 mg/kg i.p. from day 10 to day 16 after immunization. EAU was evaluated by histopathology on the eye collected at the end of the treatment period.

Major Findings

Treatment with NPC produced a dose-dependent inhibition of endotoxin-induced uveitis (EIU). At the dosages of 12, six, and three mg/kg, there was a dose-dependent reduction in the leukocyte count in the aqueous humor, accompanied by a parallel decrease in the protein concentration. Histological examination also showed a reduction in the inflammatory infiltrate of the iris and ciliary body. NPC was also effective in preventing the induction of EAU. In a representative experiment, four out of 14 NPC-treated rats developed mild EAU with an average severity grading of 1.37, whereas 10 out of 14 vehicle-treated rats developed EAU at a disease severity of 3.5.

Significance to Biomedical Research and the Program of the Institute

We conclude that the inhibition of neutrophil recruitment by NPC 15669 is effective in preventing intraocular inflammation. The treatment is effective even when instituted very late in a T-cell mediated disease such as EAU. This finding suggests that the recruitment of PMN leukocytes plays a determining role in the dynamic of intraocular inflammation.

Proposed Course

The mechanism of action of NPC 15669 in uveitis will be studied. The method will consist of measuring the expression of CD-18 adhesion molecule on the surface of inflammatory cells, stimulated *in vitro* and *in vivo* in the presence or absence of the drug.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Cheung MK, Dastgheib K, Chan C-C, Roberge FG: Inhibition of PMN recruitment by NPC 15669 prevents endotoxin induced uveitis. *Invest Ophthalmol Vis Sci* 35(4):1684, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00299-01 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of the Role of Nitric Oxide in Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	François G. Roberge	M.D.	Visiting Scientist	LI, NEI
Others:	Seiji Hayashi	M.D.	Volunteer Fellow	LI, NEI
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	Margaret Cheung	M.D., Ph.D.	Senior Staff Fellow	LI, NEI
	NamTram Pham	M.D.	Volunteer Fellow	LI, NEI
	Kourosh Dasthgieb		Visiting Fellow	LI, NEI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Immunology

SECTION

Clinical Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.9	1.9	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have studied the role of nitric oxide (NO) in two types of uveitis: (1) Anterior uveitis represented by the model of endotoxin-induced uveitis (EIU) in the rat and (2) toxoplasma retinochoroiditis using a mouse model. In EIU, we have found NO levels that peak in the anterior chamber of the eye 2 hours before cellular infiltration and a sharp rise in protein exudation. Inhibition of NO production with a structural analog of L-arginine prevented the induction of EIU. In contrast, inhibiting NO synthesis during infection with *Toxoplasma gondii* caused an exacerbation of the disease. Spleen cell cultures from infected mice produced a large amount of NO. NO production was associated with a reduced viability and proliferation of the lymphocytes to toxoplasma antigen. The lymphocyte proliferative response was restored by blocking NO production. In conclusion, it appears that NO has diverse, even opposing roles in uveitis, depending on the type and mechanism of the inflammatory process involved.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

(1) Measure the production of nitric oxide (NO) in the eye in the course of uveitis induced by endotoxin.

(2) Evaluate the effect of inhibiting the production of NO on the evolution of endotoxin-induced uveitis (EIU).

(3) Examine the effect of inhibiting NO production on the evolution of *Toxoplasma gondii (T. gondii)* infection in mice.

(4) Evaluate the role of NO in the immune response against *T. gondii.*

Methods

EIU. EIU was induced in Lewis rats with a subcutaneous injection of lipopolysaccharide (LPS) at 300 μg/kg. Aqueous humor was collected from groups of five rats every two hours for the first 12 hours, at 16 hours, and 24 hours. NO levels in the aqueous humor were measured by a colorimetric assay based on the Griess reaction. Protein levels and the number of leukocytes were also determined. In some experiments, rats were treated by intraperitoneal injections with N^G-nitro-L-arginine methyl ester (L-NAME), an L-arginine analogue acting as a specific inhibitor of NO synthesis. The aqueous humor of one eye was collected after 24 hours, and the contralateral eye was examined by histopathology.

Toxoplasmosis. Infection was induced in C57bl/B6 mice by i.p. injection of 10-20 cysts of *T. gondii* strain ME49. Aminoguanidine (AMG), an inhibitor of NO synthase, or vehicle control was administered i.p. every eight hours at 35 mg/kg. At the end of the second week, the eyes and brain were collected for histopathological examination. In other experiments, the spleens from infected and normal mice were collected two, four, and six weeks after infection and the cells stimulated with toxoplasma antigen. The proliferative response was measured by ^{3}H-thymidine incorporation in the presence or absence AMG at 1 mM. In parallel cultures, the level of NO produced was measured by a colorimetric diazotization assay of nitrite accumulated in the supernatant. Interferon gamma (IFN-γ) and prostaglandin E2 (PGE2) were also measured by specific enzyme-linked immunosorbent assay.

Major Findings

In EIU, the analysis of the aqueous humor after LPS injection showed a sharp peak of NO at eight hours, followed two hours later by a rise in protein and cell entry into the eye. Treatment of rats with L-NAME markedly reduced the level of NO and inhibited the induction of ocular inflammation by LPS. The aqueous humor protein exudate decreased by 73 to 82 percent, and the cellular infiltration was abrogated. The histopathological examination of the eyes also showed a similar inhibition of iris and ciliary body tissue infiltration in the treated rats.

The inhibition of NO production with AMG in mice infected with *T. gondii* resulted in the exacerbation of disease. On histopathological examination, the eyes of the infected mice treated with AMG showed increased inflammation in the retina, the choroid, and the vitreous compared with the infected controls. The brain showed an increased number of toxoplasma tachizoites infiltrating the tissue accompanied by a marked inflammation in AMG-treated animals. In addition, there was an accelerated evolution toward intracellular cysts formation. When spleen cells from infected mice were cultured in the presence of toxoplasma antigen, there was a negative effect on the survival and proliferation of the lymphocytes. This effect was correlated with high levels of IFN-γ, NO, and PGE2 production. The presence of AMG in the culture medium reestablished the lymphocyte proliferative response. Preventing PGE2 secretion with indomethacin also increased this response in proportion to an effect on NO production.

Significance to Biomedical Research and the Program of the Institute

In conclusion, it appears that NO has diverse opposing effect in uveitis depending on the type and mechanism of the inflammatory process involved. In anterior uveitis, the use of NO inhibitors could lead to improved therapy. In toxoplasmosis, however, it would be indicated to avoid drugs that inhibit NO synthesis. An important implication of these observations concerns the management of toxoplasma uveitis. It is a common practice to add corticosteroids to the antibiotic regimen in severe sight-threatening toxoplasmosis. In view of the negative regulation of NO production by corticosteroids, the use of antiinflammatory drugs devoid of effect on the NO synthase should be considered.

Proposed Course

Research will be directed at identifying ocular cells that may produce NO. Finding such cells in the anterior segment of the eye could lead to a new therapeutic approach in the treatment of uveitis.

We will also study the kinetics of NO production *in vivo* in mice infected with *T. gondii.* In particular, we will try to identify cells expressing NO synthase in the eye and the brain during infection. Later we will compare the levels expressed in sensitive and resistant strains with toxoplasmosis. We will also study the possible effect of NO inhibition on triggering reactivation of chronic latent toxoplasmosis.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Hayashi S, Gazzinelli R, Chan C-C, Pham N, Roberge FG: In vivo inhibition of nitric oxide enhances ocular and CNS inflammation in murine toxoplasmosis. *Invest Ophthalmol Vis Sci* 35(4):1685, 1994.

Parks DJ, Cheung MK, Chan C-C, Roberge FG: The role of nitric oxide in uveitis. *Arch Ophthalmol* 112:544, 1994.

Roberge FG, Hayashi S: Nitric oxide is responsible for the inhibition of lymphocyte proliferation in Toxoplasma gondii infection. *Invest Ophthalmol Vis Sci* 35(4):1685, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00279-03 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Immunosuppressants for the Treatment of Uveitis in Animal Models

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	François G. Roberge	M.D.	Visiting Scientist	LI, NEI
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	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Dan Martin	M.D.	Senior Staff Fellow	LI, NEI
	Margaret Cheung	M.D., Ph.D.	Senior Staff Fellow	LI, NEI
	David Parks	M.D.	Senior Staff Fellow	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Clinical Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0	0	0.0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

THIS PROJECT HAS BEEN TERMINATED.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00301-01 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ocular Gene Transfer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Karl G. Csaky	M.D., Ph.D.	Medical Officer	LI, NEI
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	Eddy Anglade	M.D.	Staff Fellow	LI, NEI
	Csaba Salaman	M.D.	Visiting Scientist	LI, NEI
	Robert Interrante	M.S.	Special Volunteer	LI, NEI

COOPERATING UNITS *(if any)*

S.U.N.Y. at Stony Brook, Stony Brook, NY (L. Taichman, M.D., Ph.D.); Department of Pediatrics, The Johns Hopkins University, Baltimore, MD (D. Valle, M.D.); Human Genome Center, NIH, Bethesda, MD (M. Blaese, M.D.); Massachusetts Institute of Technology, Department of Chemical Engineering, Boston, MA (D. Mooney, Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section of Ocular Gene Therapy

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.75	2.25	0.5

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [X] (b) Human tissues [ ] (c) Neither
[ ] (a1) Minors
[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

An ocular gene therapy section is being developed that emphasizes the study of direct ocular gene transfer using E1A-deficient adenovirus constructs, the transfer of genes *ex vivo* into isolated retinal pigment epithelial cells prior to their transplantation, and the use of ornithine aminotransferase (OAT) transfected keratinocytes to treat patients with gyrate atrophy.

A mutant of transforming growth factor beta-1 (TGF-β), which is secreted in an active form, has been placed into an adenovirus construct. Studies involving transduction of retinal pigment epithelial cells have shown that these cells can be efficiently transducted at a low multiplicity of infection and are capable of producing high levels of a biologically active TGF-β. Adenovirus-TGF-β has also been injected into the anterior chamber of rats. Immunohistochemical analysis of these eyes has suggested the presence of secreted TGF-β.

Human retinal pigment epithelial cells (RPE) have been transfected with plasmids containing beta-galactosidase (β-gal). Studies are underway to examine the optimal modes of transfection. Parallel studies are being done with isolated rat RPE cells. Both cell lines are capable of growth on thin synthetic basement membrane polymers. These polymers are transplanted, as a single cell layer, into the subretinal space of rats.

OAT has been successfully transfected into OAT-deficient fibroblasts (CHO cells) and into keratinocytes, isolated from patients with gyrate atrophy. Studies are presently underway to examine ways to increase OAT levels and activity in these cells.

Project Description

Objectives

An ocular gene therapy section is being established, the objective of which is to apply molecular biology techniques in the treatment of human ocular disease. Major areas of interest include direct ocular gene transfer using E1A-deficient adenoviruses, transfection of retinal pigment epithelial cells *ex vivo* before their transplantation and the transfection of ornithine-δ-aminotransferase (OAT) into keratinocytes from patients with gyrate atrophy (GA). Optimization of expression of OAT in cultured keratinocytes from patients with GA will allow us to determine if transfected keratinocytes can be used as a sufficient source of OAT to treat this disorder.

Methods

Using standard cloning techniques, adenovirus constructs are being synthesized. Using polymerase chain reaction, Northern, Western, Southern, and enzyme-linked immunosorbent assay (ELISA) methods, sequence and expression data from various adenovirus constructs such as transforming growth factor (TGF)-β and OAT are being generated.

Site-directed mutagenesis of the OAT gene and alteration in the promoters and their proximity to translational start sites are being performed. This will allow us to identify OAT constructs that, when transfected into keratinocytes, will most efficiently degrade circulating ornithine. Standard tissue-culture techniques and the study of synthetic basement membrane polymers are used to study the transplantation of retinal pigment epithelium (RPE) into rodent eyes. This approach will allow the determination of the usefulness of these techniques in the treatment of animals with ocular neovascularization and diabetic macular edema.

Major Findings

Human RPE cells appear to be efficiently transduced by E1A-deficient adenovirus. By using a lac-Z/E1A-adenovirus construct, human RPE were shown to express β-galactosidase activity in 100 percent of transduced cells.

E1A-deficient adenovirus constructs with TGF-β have been produced. As a result of site-directed mutagenesis, the TGF-β construct is secreted in an active form as a 24 kDa protein. This is unlike endogenous TGF-β1, which is secreted as a 90 to 110 kDa protein and requires activation by a latent factor to become biologically active. We have been able to show that the efficient transduction of human RPE can be readily achieved with the use of E1A-deficient adenovirus-TGF-β. Even at low multiplicity of infection, studies have shown that expression of active TFG-β can be achieved to pharmacological levels. TFG-β can be quantified by an ELISA assay, has been shown to be biologically active, and correlates with ribonucleic acid expression. The virus also has been injected into the anterior chamber of rats. At one to seven days following injection, immunohistochemical staining has demonstrated the presence of TGF in the corneal and iris stroma. Injection with null E1A-deficient adenovirus lacked TGF-β expression, suggesting specificity of the expression.

Human and transformed rat RPE have been isolated and grown in culture. Transfection of cytomegalovirus (CMV)-lacZ constructs into these cell lines, with lipofectant or calcium phosphate, have yielded transfection efficiencies of 20 to 30 percent. Transfection efficiency, into human RPE, was increased by contransfecting attenuated adenovirus with lipofectamine. This achieved a transfection efficiency of 90 percent. Both cell types have been grown, as a monolayer, on a 20 μm thick polylactic/polyglycolic polymer. Transplantation of these sheets into the subretinal space of rats is now being performed.

Keratinocytes from patients with GA have been isolated and grown in culture. They exhibit low levels of endogenous OAT activity. Various OAT constructs have been generated. Expression of full-length OAT inserts, under a CMV or long terminal repeat (LTR) promoter, have been tested in CHO cells. Under LTR control, this construct yielded a twentyfold higher OAT activity than mock transfected cells. However, when transfected into the isolated keratinocytes, the increase was only threefold. Several OAT constructs were made with varying lengths of untranslated regions and portions of the mitochondrial leader to the protein removed. Studies are now underway to determine which of these constructs is most efficient at expressing biologically active OAT.

Significance to Biomedical Research and the Program of the Institute

Direct ocular gene transfer has the potential for treatment in hereditary ocular diseases. In diseases such as GA where a deficiency of OAT is responsible for the clinical findings, gene therapy offers the possibility of cure. From a biological perspective, direct gene transfer into ocular tissues allows the study of the biology of overexpression of certain proteins into the eye. When animal models of disease are available, the use of direct gene transfer with adenovirus or transplantation of transfected RPE cells can identify critical proteins in the amelioration of these diseases. For example, TGF-β has been proposed as a downregulator of the immune response. By overexpressing TGF-β in animal models of uveitis, direct examination of this hypothesis can be obtained.

In the event that keratinocytes from patients with GA transfected with a construct of OAT exhibit sufficient OAT activity to degrade significant serum ornithine, these cells will then be replaced onto the patient's skin. If serum ornithine levels fall, this will offer the first gene therapy for an ocular disease.

Proposed Course

The biology of overexpression of TGF-β in rodent models of uveitis will be further examined. Various other inflammatory cytokines and inhibitors such as interleukin (IL)-10 and IL-1 receptor antagonist will also be investigated in this fashion. Further investigation of the effect of transduction by adenovirus on RPE cells will be performed. Subretinal injections of adenovirus constructs will result in transduced RPE cells overexpressing selected proteins *in vivo*. The biologic significance of this overexpression in the subretinal space can then be examined.

We will examine RPE cells, transplanted in a monolayer in the subretinal space, to determine if these cells are able to function normally. The kinetics of gene expression from these cells, transfected *ex vivo*, will also be determined.

The development of other mutants of OAT will continue. Once assayed for biological activity in degrading ornithine, these constructs will be examined for expression and activity in cultured keratinocytes from GA patients. If sufficient OAT is produced that is capable of degrading adequate amounts of serum ornithine, further steps will be taken to replace these keratinocytes in the GA patients. Adenovirus constructs containing OAT will be created, and the study of their expression following ocular injections in mouse OAT knockout models of GA will be accomplished.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00268-04 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Diagnosis and Treatment of Human Uveitis and AIDS-Related Ocular Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Scott M. Whitcup	M.D.	Staff Medical Officer	CB, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Chi-Chao Chan	M.D.	Medical Officer	LI, NEI

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Department of Medicine, The Johns Hopkins University, Baltimore, MD (David R. Moller, M.D.)

LAB/BRANCH

Clinical Branch/Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither
[ ] (a1) Minors
[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The title of this project has been changed from "The Diagnosis and Treatment of Human Uveitis" to "The Diagnosis and Treatment of Human Uveitis and AIDS-Related Ocular Disease," to reflect the expanded scope of the project to include the study of ocular disease in patients with AIDS. In addition, this project was previously listed in the Laboratory of Immunology but is now listed in the Clinical Branch. The goal of this project is to develop improved methods for diagnosing and treating human uveitis and AIDS-related ocular disease. This project encompasses clinical trials evaluating new diagnostic and therapeutic approaches and immunologic and histologic studies on blood and tissue specimens obtained from patients.

A number of studies have focused on improving diagnostic tests for uveitis. Examination of lacrimal gland and conjunctival biopsies from patients with sarcoidosis has demonstrated lymphocytic infiltration and specific T-cell receptor repertoires despite a lack of granuloma formation, and these results should help improve the diagnostic yield of biopsies for the diagnosis of sarcoidosis. We have also shown that lumbar puncture can miss malignant involvement of the leptomeninges in patients with intraocular lymphoma and that ventricular taps are needed.

The Clinical Branch is involved in a number of studies designed to improve the treatment of uveitis and ocular malignancy. A prospective, double-masked, randomized study of acetazolamide for uveitis-associated cystoid macular edema has just completed enrollment, and data will be analyzed this year. In addition, we completed a pilot study examining the efficacy and toxicity of a chemotherapy regimen for therapy of patients with central nervous system lymphoma involving the brain or eye. A complete response was obtained in seven of eight patients; the other patient had a partial response. A clinical trial evaluating a heparin surface-modified intraocular lens in patients with uveitis is under way, and a natural history study of patients with uveitis and good vision is in progress.

Finally, the Clinical Branch is involved in studies with ocular complications related to AIDS. We are retrospectively reviewing the ophthalmologic examinations of 550 AIDS patients to improve diagnosis of ocular complications such as infection. In addition, we are participating in an open-label study of cidofovir for the treatment of resistant cytomegalovirus (CMV) retinitis in patients with AIDS.

Project Description

Additional Personnel

Emily Chew	M.D.	Visiting Scientist BEP, NEI
Frederick Ferris, III	M.D.	Chief, Clinical Trials Branch BEP, NEI
George P. Chrousos	M.D.	Medical Officer NICHD/DEB
George Mastorakos	M.D.	Visiting Scientist NICHD/DEB
Igal Gery	Ph.D.	Deputy Chief LI, NEI
Susan Mellow	R.N.	Nurse Specialist CB, NEI
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Clinical Protocol Numbers

87-EI-0104
91-EI-0030
91-EI-0139
92-EI-0070
92-EI-0108
92-EI-0138
92-EI-0157
94-EI-0189

Objectives

The title of this project has been changed from "The Diagnosis and Treatment of Human Uveitis" to "The Diagnosis and Treatment of Human Uveitis and AIDS-Related Ocular Disease," to reflect the expanded scope of the project to include the study of ocular disease in patients with acquired immunodeficiency syndrome (AIDS). The goal of this study is to develop better methods for the diagnosis and treatment of human uveitis and ocular disease associated with AIDS. We are also interested in defining the pathophysiology of inflammatory eye diseases by performing immunologic and histologic studies on tissue specimens and blood samples obtained from patients.

Methods

Diagnosis of Uveitis and Malignancy

(1) To improve the diagnostic yield of conjunctival and lacrimal gland biopsies for sarcoidosis, tissue specimens are examined using immunohistochemical staining and polymerase chain reaction (PCR). Conjunctival and lacrimal gland biopsies are performed and snap frozen in OCT from patients with known sarcoidosis.

Immunohistochemical staining is performed using primary monoclonal antibodies against T-cell markers, T-cell receptors, Kveim antigen, and various interleukins. The cell receptor repertoire is also studied using PCR techniques. Results will be compared with biopsies from patients with other uveitic conditions such as Behçet's disease to determine the specificity of these results.

(2) Intraocular lymphoma often masquerades as an idiopathic uveitis that delays the start of appropriate therapy. We continue to prospectively collect data on patients with intraocular lymphoma to improve diagnosis in these patients.

(3) Samples of aqueous humor, iris, and vitreous are obtained from patients undergoing ocular surgery for uveitis. Samples will be assayed for both cytokines and cell-adhesion molecules and compared with samples obtained from patients undergoing routine cataract surgery in an attempt to identify immunologic markers for ocular inflammation.

(4) Animals with experimental autoimmune uveitis develop an associated pinealitis. It is not known whether patients with human uveitis also have associated inflammation of the pineal gland. We are examining patients with uveitis for the presence of pinealitis using magnetic resonance imaging (MRI).

Treatment of Uveitis of Malignancy

(1) The efficacy of acetazolamide for the treatment of uveitis-associated macular edema

is being evaluated in a masked crossover study comparing acetazolamide with placebo. Visual acuity and the height of the macular edema measured on fluorescein angiography will be used as primary endpoints.

(2) We are investigating the treatment of patients with non-Hodgkin's lymphoma involving the brain or eye. The NEI is participating in a pilot study to evaluate the efficacy and toxicity of a chemotherapy regimen for primary therapy of patients presenting with central nervous system lymphoma involving the brain or eye. High-dose methotrexate and leucovorin rescue is used in combination with thiotepa, vincristine, dexamethasone, and intrathecal cytarabine, and G-colony stimulating factor (G-CFS) when needed. Radiation therapy is deferred until patients show evidence of disease progression. Patients are followed by clinical ophthalmologic examinations as well as MRI and nuclear magnetic resonance spectroscopy. Laboratory correlates, including immunohistochemistry and viral sequence detection, are performed on available tissue. The data obtained from this study will be used to design a more inclusive study of chemotherapy in ocular and central nervous system lymphoma.

(3) The purpose of this project is to evaluate the ability of a heparin surface-modified intraocular lens to reduce the incidence and severity of postoperative inflammation in patients with uveitis undergoing cataract surgery. Patients with a history of uveitis and cataracts, scheduled for cataract extraction with placement of an intraocular lens, will be randomized to receive either a heparin surface-modified intraocular lens or a nonmodified lens. The primary endpoint of the study is inflammatory cell deposits in the intraocular lens. Secondary endpoints will include other measures of ocular inflammation and visual acuity. Eighty patients will be recruited for the study.

(4) The clinical course of patients with Behçet's disease who were treated with cyclosporine alone was compared retrospectively with patients who were treated with combined cyclosporine and prednisone.

AIDS

(1) We are retrospectively reviewing the ophthalmologic examinations of 550 patients with AIDS in an attempt to improve the diagnosis of cytomegalovirus (CMV) retinitis. Information, including patient symptoms, visual acuity, and ocular examination data and CD-4 T-lymphocyte counts, will be analyzed to establish criteria for screening patients for ophthalmologic disease. We are also using laser interferometry to measure anterior chamber flare to determine whether this device can provide a sensitive and specific test for CMV retinitis in patients with AIDS.

(2) The NEI is participating in an open-label, randomized, multicenter study of the safety and efficacy of cidofovir for the treatment of relapsing CMV retinitis in patients with AIDS. Patients with evidence of CMV retinitis progression while receiving ganciclovir and/or foscarnet therapy will be randomly assigned to one of two dose levels of cidofovir. Patients will undergo ophthalmologic examination at baseline and at regular intervals thereafter. The primary endpoints are safety and time to progression of CMV retinitis.

(3) Eyes obtained at autopsy are studied in an attempt to understand the pathophysiology of AIDS-related ocular disease. In addition, the clinical course of all patients with AIDS and ocular disease are prospectively studied.

Major Findings

Diagnosis of Uveitis and Malignancy

(1) We have recruited nine patients with biopsy-proven sarcoidosis into the protocol examining conjunctival and lacrimal gland biopsies. None of the biopsies had granuloma formation diagnostic for sarcoidosis. Nevertheless, substantial areas of lymphocytic infiltration were present on most biopsies, and we

are assessing the T-cell receptor repertoire in these specimens.

(2) Evaluation of the diagnostic data from patients with intraocular lymphoma has shown that despite the absence of malignant cells in the cerebral spinal fluid obtained by lumbar puncture, malignant cells were demonstrated in a sample simultaneously obtained from an Omaya reservoir placed in the ventricles of five patients. The data suggest that malignant leptomeningeal involvement may be missed by lumbar puncture alone.

(3) Studies on the levels of soluble intercellular adhesion molecule 1 in the serum, iris specimens, and aqueous of patients with uveitis are in progress.

(4) We continue to recruit patients in the protocol using MRI to evaluate the pineal gland in patients with uveitis.

Treatment of Uveitis and Malignancy

(1) We have completed enrollment of patients in the crossover study of acetazolamide for the treatment of uveitic cystoid macular edema. The last patient is completing the study course; the randomization will be unmasked, and the data will be analyzed during the next year.

(2) Eight patients were enrolled in this pilot study and recruitment was completed in November 1993. Seven of eight patients had complete remission on this protocol; one patient had a partial response. Definite grade IV toxicity occurred in only one patient. The phase II trial examining chemotherapy for the treatment of central nervous system lymphoma has just started recruiting patients.

(3) Seven patients have been enrolled in the protocol examining the heparin surface-modified intraocular lens in patients with uveitis.

(4) We reviewed 19 patients with severe ocular Behçet's disease treated with combined cyclosporine and corticosteroid therapy. Previous treatment with corticosteroids alone failed to control the uveitis in all patients. Ten patients were given cyclosporine therapy alone (mean dosage, 8.6 mg/kg of body weight per day), and nine patients were given lower dosages of cyclosporine (mean dosage, 6.2 mg/kg of body weight per day) in combination with prednisone (mean dosage, 29.4 mg per day). The mean followup on therapy was 51 months. After three months of therapy, a trend toward greater improvement in visual acuity was noted in patients treated with combined cyclosporine and prednisone compared with those receiving cyclosporine alone (17.8 letters vs. 10.2 letters, $p = .24$); after one year, little difference was observed in the improvement between the two groups (5.8 letters vs. 3.3 letters, $p = .80$). However, a trend toward greater renal toxicity was seen in patients treated with cyclosporine alone after both three months and one year of therapy. Because of either a suboptimal therapeutic response or adverse effects, all patients treated with cyclosporine alone at baseline had prednisone added to their regimen after a mean time of 23.5 months. Overall, visual acuity remained stable or improved in 28 of 37 eyes (75.7 percent) over the course of therapy. The data suggest that combined cyclosporine and prednisone therapy is an effective treatment for Behçet's uveitis and may be less toxic than therapy with cyclosporine alone.

AIDS

(1) Laser interferometry has shown increased flare in the anterior chambers of patients with CMV retinitis and may be a useful screening test of ocular inflammatory disease in patients with AIDS.

(2) Recruitment into the trial of cidofovir for CMV retinitis has started.

(3) We have found an increased severity of CMV retinitis in children with AIDS as compared with adults. This increased severity appears to be related to a delay in diagnosis because children are less apt to complain of visual symptoms and seek ophthalmologic evaluation than adults. Increased vigilance in

screening children with AIDS for the development of ocular complications appears warranted. We also described atypical findings of mild peripheral retinopathy in a patient with acute retinal necrosis following chicken pox, which may have been related to early acyclovir therapy.

Significance to Biomedical Research and the Program of the Institute

Uveitis accounts for about 10 percent of the visual impairment in the United States. A major goal of the NEI is to improve the methods for diagnosing and treating uveitis in an attempt to preserve useful vision in patients with inflammatory eye disease. In addition, many patients with AIDS develop severe, sight-threatening ocular disease. CMV retinitis is the most common cause of impaired visual acuity in patients with AIDS, occurring in 10 to 30 percent of AIDS patients. An important goal of the NEI is to improve our methods of diagnosing AIDS-related eye disease and to develop and test new therapies for these disorders.

Proposed Course

We will continue to recruit patients with intraocular lymphoma, uveitis, and AIDS-related ocular disease into the clinical trials detailed earlier. A new protocol that looks at novel therapeutic approaches for CMV retinitis will be started during the next year. We have completed the enrollment of patients into the crossover study of acetazolamide for uveitic cystoid macular edema and will start the analysis of the data. In addition, we have completed our collection of ophthalmologic data from the 550 patients with AIDS. Our plan is to analyze the compiled data and issue recommendations for the screening of patients with AIDS for ocular disease.

NEI Research Program

Retinal Diseases—Inflammatory Diseases, Cancer

Publications

Callanan DG, Cheung MK, Martina DF, de Smet MD, Whitcup SM, Nussenblatt RB: Outcome of uveitis patients treated with long-term cyclosporine. *Invest Ophthalmol Vis Sci* 35(suppl):2094, 1994.

Friedman SM, Mames RN, Whitcup SM: Acute retinal necrosis after chickenpox in a patient with acquired immunodeficiency syndrome. *Arch Ophthalmol* 111:1607-1608, 1993.

Whitcup SM, Salvo EC Jr, Nussenblatt RB: Combined cyclosporine and corticosteroid therapy for sight-threatening uveitis in Behçet's disease. *Am J Ophthalmol* 118:39-45, 1994.

Whitcup SM, Nussenblatt RB: Treatment of autoimmune uveitis. *Ann N Y Acad Sci* 696:307-318, 1993.

Whitcup SM, de Smet MD, Rubin BI, Palestine AG, Martin DF, Burnier M Jr, Chan C-C, Nussenblatt RB: Intraocular lymphoma: Clinical and histopathologic diagnosis. *Ophthalmology* 100:1399-1406, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00222-09 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunopathology in Eyes With Experimental and Clinical Ocular Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	Bo Peng	M.D.	Visiting Fellow	LI, NEI
	Deborah Luyo		Technician	LI, NEI
	Scott M. Whitcup	M.D.	Associate Director	CB, NEI
	Charles E. Egwuagu	Ph.D.	Medical Officer	LI, NEI
	François G. Roberge	M.D.	Visiting Scientist	LI, NEI
	Rachel R. Caspi	Ph.D.	Visiting Scientist	LI, NEI
	Igal Gery	Ph.D.	Deputy Chief	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 5.0	PROFESSIONAL: 4.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The identity and topographic localization of immunocompetent cells and the alteration of surface markers on ocular resident cells in animals with various experimental uveitis were analyzed by immunohistochemical studies and *in situ* hybridization. Previously, we have demonstrated that T lymphocytes were the predominantly infiltrating cells in experimental autoimmune uveoretinitis (EAU), yet both macrophages and polymorphonuclear neutrophils were the predominantly infiltrating cells in endotoxin-induced uveitis (EIU). Cytokines (e.g., interferon gamma), inflammatory mediators (e.g., nitric oxide), and some retinal proteins (e.g., S-antigen (S-Ag)) play an important role in the immunopathogenesis of EAU and EIU. Agents that modulate them can alter the immunopathology of the experimental model. For example, prolonged corticosteroid therapies enhance S-Ag expression in nonretinal ocular tissues of rats with EAU.

Several new animal models, including experimental melanin-protein-induced uveitis (EMIU), experimental blepharitis, murine allergic conjunctivitis, and murine toxoplasmosis, have been developed and studied. They represented different entities of uveitis in humans. EMIU closely resembles Vogt-Kayanagi-Harada syndrome and sympathetic ophthalmia; experimental blepharitis induced by immunization of monoclonal antibody of Id16/6 resembles idiopathic blepharitis; murine allergic conjunctivitis induced by compound 48/80 resembles allergic conjunctivitis; murine toxoplasmosis infected with *T. gondii* resembles acquired ocular toxoplasmosis.

Specimens from human ocular tissues with various diseases, including uveitis, retinal and corneal diseases, tumors, and metabolic genetic disorders, are studied using immunohistochemical and *in situ* hybridization techniques as well as light and electron microscopic examinations. In uveitis, immunocompetent cells and lymphokines besides the stimulators (e.g., infectious organisms) are valuable adjuncts to the clinical diagnosis and the understanding of pathogenesis of the diseases. In nonuveitic conditions, alteration of cellular membrane surface markers and intracytoplasmic organelles of the ocular resident cells (e.g., crystalline inclusions in Beitti's crystalline dystrophy; B-cell marker in intraocular lymphoma) may reflect cellular damage and abnormalities in these diseases. Elucidating the immunopathological role of the relationships between infiltrating inflammatory or malignant cells and other resident cells in the clinical behavior of various diseases will increase our understanding of human ocular disorders and provide better treatment.

Project Description

Objectives

This program is designed to evaluate the clinical manifestation, histopathology, and immunopathology of the ocular tissue when experimental uveitis (experimental autoimmune uveitis [EAU], endotoxin-induced uveitis [EIU], experimental melanin-induced uveitis [EMIU], murine toxoplasmosis, etc.) are induced and/or modulated by different immunosuppressive agents in various animal species. Ocular tissues obtained from patients with various diseases, including inflammatory and noninflammatory disorders, are all included. The infiltrating inflammatory cells, ocular resident cells and their products, cytokines, and metabolites are examined. These findings will help us understand ocular inflammation and the pathogenesis of each disease examined in humans.

Methods

Clinical examinations include flashlight, slit-lamp and fundus examination, under the dissecting microscopes of experimental animals and patients. Pathological examinations include routine histologic techniques for light and electron microscopy, immunofluorescence and avidin-biotin-peroxidase complex method, and *in situ* hybridization techniques. Enzyme-linked immunosorbent assay technique is also performed for cytokines or protein analysis of the serum and intraocular fluids.

Major Findings

We have continued to study the immunopathology of various inflammatory cells and ocular resident cells in different experimental models of uveitis. We have demonstrated that higher levels of S-antigen (S-Ag) and its messenger ribonucleic acid (mRNA) are expressed in nonretinal ocular cells (the lens, the ciliary body, and the trabecular meshwork) of rats with EAU, in particular those that also received long-term corticosteroid treatment. This experimental result supports our previous finding of S-Ag and its mRNA detected in irises of some uveitic patients who received long-term steroids.

Several new experimental models for uveitis in humans have been investigated by us. EMIU is induced by immunization with bovine choroidal and retinal pigment epithelium (RPE) melanin protein and is characterized by bilateral, recurrent iridocyclitis and choroiditis. In EMIU, like EAU, the main infiltrating cells are T lymphocytes of CD-4 cells seen in the early stage and CD-8 cells seen in the late stage of the disease. Expressions of adhesion molecules and major histocompatibility (MHC) class II antigens are observed on ocular resident cells one to two days before ocular inflammation (eight to 10 days postimmunization). However, unlike EAU, recurrence occurs one month later.

Acquired ocular toxoplasmosis is developed by the infection of an avirulent strain of *Toxoplasma gondii* (*T. gondii*) (ME49) in C57BL/6 mice. Focal ocular inflammation and RPE involvement are observed after 15 days of infection. Four weeks after infection, ocular inflammation becomes stable and rare cysts can be found. Treatment of mice with antibodies (CD-4 plus CD-8) or cytokines (interferon gamma [IFN-γ] or tumor necrosis factor alpha [TNF-α]) results in a marked increase of ocular inflammation associated with the presence of the parasite.

In the study of transgenic mice with constitutive expression of IFN-γ in the eye, we have reported that the growth of the eye is affected, resulting in microphthalmia, cataracts, arrest of retinal differentiation, and enhancement of the expression of MHC class II. In a study of the role of nitric oxide, we have demonstrated that competitive blocking of NO formation with the L-arginine analogue is sufficient to inhibit the induction of EIU.

Using immunopathological techniques, we examine ocular tissues obtained from patients with various ocular diseases to help visualize the pathology and the kinetics of the specific disease process. The findings provide useful

information for understanding the pathological mechanisms of the disease, determining the diagnosis, and guiding the subsequent management of the patient. Iris biopsies from patients with uveitis and cataracts were studied. All except one specimen from uveitic patients had T lymphocyte and macrophage infiltration. The consistent spatial correlation between the presence of T cells and IFN-γ as well as between the presence of macrophages and defensin are observed.

Retinal necrosis, neovascularization, marked chorioretinal inflammation, particularly of T lymphocytic infiltration, and the absences of bradyzoites are the characteristic findings in fetal eyes infected with *T. gondii.* Polymerase chain reaction (PCR), a sensitive and specific technique to identify infectious agents, and immunohistochemistry are helpful for the diagnosis of ocular toxoplasmosis.

Twelve cases of intraocular lymphoma diagnosed at the NEI between 1984 and 1992 were retrospectively reviewed. All were non-Hodgkin's large B cell lymphoma of the central nervous system. The prompt, appropriate handling of specimens and review by an experienced cytopathologist are critical to the diagnosis of intraocular lymphoma. Malignant cells often are present in the vitreous before and/or in the cerebral spinal fluid. Multiple vitrectomies and lumbar punctures may be necessary before the correct diagnosis is made.

Didanosine (DDI), used in the treatment of acquired immunodeficiency syndrome, is associated with toxic retinopathy. We have studied the first pathological report and demonstrated numerous membranous lamellar inclusions and cytoplasmic bodies in the affected RPE cells. These data show that the DDI retinopathy results from the pathology of RPE cells.

We evaluated three affected members of a Chinese-American family with Bietti's crystalline retinopathy. Crystalline lysosomal materials are observed in lymphocytes and skin fibroblasts of these patients. The advanced panchorioretinal atrophy with crystals and complex lipid inclusions in the choroidal fibroblast was first documented in this study.

Significance to Biomedical Research and the Program of the Institute

Immunopathological findings on experimental uveitides have provided information on various inflammatory cells and ocular resident cells during the process of ocular inflammation. This information helps us to better understand the mechanisms of uveitis and select or evaluate novel pharmacological agents as well as provide suitable therapeutic intervention of uveitis in humans. Studies of ocular tissues obtained from patients with various disorders have enabled us to gain information on the pathogenesis, diagnosis, and management of these ocular diseases.

The finding that corticosteroids enhance S-Ag expression in nonretinal ocular tissues of rats with EAU may have clinical implication. The alteration of protein (such as S-Ag) expression on the lens, the ciliary body, and the trabecular meshwork may contribute to the ocular side effects induced by prolonged corticosteroid therapy in patients.

Melanin protein is capable of inducing autoimmune uveitis, resembling noninfectious recurrent iridocyclitis and choroiditis in humans. EMIU is another useful model for the study of uveitis such as Vogt-Kayanaki-Harada Syndrome and the evaluation of immunomodulating agents.

Murine toxoplasmosis is a practical model for us to understand the respective roles of *T. gondii* proliferation and immune mechanisms involved in the pathogenesis of acquired ocular toxoplasmosis. T lymphocytes as well as TNF-α and IFN-γ are crucial elements in controlling parasite growth. In immunocompromised hosts such as AIDS patients, ocular lesions can be more severe and result from parasite proliferation rather than from an autoimmune process.

Early indicators of ocular toxoplasmosis in the fetus are infiltrating T cells and the absence of tachyzoite antigens in the lesions. PCR for the diagnosis of ocular toxoplasmosis is helpful.

The study demonstrates that T cells and macrophages are part of the inflammatory response in uveitis and that they secrete IFN-γ and defensin, respectively. These findings may have implications for the treatment of ocular uveitis. Antibody or specific immunomodulatory therapy that abrogates the effects of cytokines such as IFN-γ or defensins may decrease the extent and severity of ocular inflammation.

Proposed Course

All experimental models, including EAU, EIU, EMIU, allergic conjunctivitis, experimental blepharitis, and murine toxoplasmosis will be studied clinically, histopathologically, and immunopathologically in different strains and species. Various pharmacological agents and the role of cytokines, enzymes, metabolites, and cellular markers will be evaluated in these models. Also, we propose continuation of the analysis of human specimens to understand their immunopathogenesis.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Anderson W, Chan C-C, Nussenblatt RB, Whitcup SM: Topical heparin inhibits compound 48/80 induced allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 35(4):1291, 1994.

Brezin AP, Kasner L, Thulliez P, Li Q, Daffos F, Nussenblatt RB, Chan C-C: Ocular toxoplasmosis in the fetus: Immunohistochemistry and DNA amplication. *Retina* 14:19-26, 1994.

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Chan C-C, Gery I, Nussenblatt RB, Mozes E, Singer DS: Periocular inflammation in mice with experimental systemic lupus erythematosus (SLE): A new experimental eye disease and its modulation. *Invest Ophthalmol Vis Sci* 35(4):1988, 1994.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00270-04 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunologic Mechanisms of Ocular Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Scott M. Whitcup	M.D.	Staff Medical Officer	CB, NEI
Others:	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Sei-Ichi Ishimoto	M.D.	Visiting Associate	LI, NEI (CRADA)

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Branch/Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The title of this project has been changed from "cell adhesion molecules in ocular inflammation" to "immunologic mechanisms of ocular disease" to reflect the broadening scope of experiments to encompass the role of cell adhesion molecules and other immunologic factors, including cytokines, in the pathogenesis of ocular diseases, particularly uveitis, ocular malignancy, and ocular disease related to AIDS. In addition, this project was previously listed in the Laboratory of Immunology but is now listed in the Clinical Branch. The goal of this project is to study the immunologic mechanisms involved in the pathogenesis of ocular inflammation and ocular malignancy and to develop and test therapies based on these data. Recently, we have concentrated on the role of cell adhesion molecules and cytokines in the development of ocular inflammatory disease. Cell adhesion molecules (CAMs) are surface proteins important for antigen sensitization and the migration of leukocytes to sites of inflammation. We are currently investigating compounds that block CAMs as a treatment for uveitis and other ocular inflammatory diseases. During the past year, we showed that a single injection with a monoclonal antibody against the CAMs, lymphocyte function-associated antigen-1 (LFA-1) and Mac-1, can inhibit the development of uveitis by more than 66 percent. In contrast, treatment with a monoclonal antibody against a third CAM, vascular adhesion molecule-1 (VCAM-1) failed to inhibit *in vivo* disease. Antibodies against these cell adhesion molecules also inhibited lymphocyte proliferation *in vitro* by up to 70 percent.

We also studied the role of interleukin-12 on the development of acute intraocular inflammation and showed that systemically administered antibody against IL-12 exacerbates the endotoxin-induced uveitis. Similarly, intraocular IL-12 inhibited disease. These data suggest that endotoxin-induced uveitis may be a Th-2-dependent disease. Finally, we investigated the effect of a number of therapeutic agents in animal models of ocular inflammation. We demonstrated that MDL 28,842, an inhibitor of S-adenosyl-L-homocysteine hydrolase, decreases the ocular inflammation in animals with experimental autoimmune uveitis (EAU). In other experiments, we showed that topically administered heparin inhibits the development of allergic conjunctivitis induced by mast cell degranulation.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Rachel Caspi	Ph.D.	Visiting Associate LI, NEI
Igal Gery	Ph.D.	Deputy Chief LI, NEI
Qian Li	M.D.	Visiting Fellow LI, NEI
R. Christopher Walton	M.D.	Senior Staff Fellow LI, NEI

Objectives

The goal of this project is to study the immunological mechanisms involved in the pathogenesis of ocular inflammation and malignancy and to develop and test new therapies for these disorders. The project has focused on investigating the role of cell-adhesion molecules and cytokines in ocular inflammation and testing new therapeutic agents using *in vitro* and *in vivo* models of ocular inflammatory disease.

Methods

Animal Models of Ocular Inflammation. Endotoxin-induced uveitis (EIU) is induced by injecting 100 μg of *Salmonella typhimurium* endotoxin into one footpad of Lewis rats or 200 μg into one footpad of C3H-Hen mice. Experimental autoimmune uveitis (EAU) in mice is induced by immunizing B10.A mice with 50 μg of interphotoreceptor retinoid binding protein in complete Freund's adjuvant, with pertussis toxin injected intraperitoneally. Finally, allergic conjunctivitis was induced in mice using topical administration of compound 48/80, a mast cell degranulating agent.

Histology and Immunohistochemistry of Ocular Inflammation. Enucleated animal eyes and human ocular tissue are immediately snap frozen and embedded in OCT. The expression of cell-adhesion molecules and presence of cytokines is then assessed with immunohistochemical staining using an avidin-biotin-peroxidase complex method on frozen sections of ocular tissue. Eyes are also embedded in methyl methacrylate, and four micron sections are examined for histologic evidence of inflammation. In the model of compound 48/80 induced allergic conjunctivitis, tarsal and bulbar conjunctiva were removed and processed for routine histology or frozen for immunohistochemical staining as described earlier.

In vitro lymphocyte proliferation assays are performed as previously detailed (*Cell Immunol* 122:251, 1989).

Major Findings

(1) In a study examining the effect of treatment with a monoclonal antibody against lymphocyte function-associated antigen (LFA)-1 and very late activation antigen (VLA)-4, we demonstrated that anti-LFA-1 antibody significantly inhibited the development of EIU. In contrast, anti-VLA-4 antibody had no effect on the development of intraocular inflammation. The data suggest that compounds blocking LFA-1 but not VLA-4 should be effective for the treatment of acute ocular inflammation. Studies *in vitro* showed that antibodies against LFA-1 and Mac-1 inhibited lymphocyte proliferation but that anti-VLA-4 antibody had less effect. These data suggest that anti-LFA-1 and anti-Mac-1 antibody may be useful in the treatment of lymphocyte-mediated ocular inflammatory disease. Finally, increased expression of cell-adhesion molecules was noted in corneal specimens with allograft failure, suggesting that antibodies against adhesion molecules may prevent rejection.

(2) We found that systemic treatment with anti-interleukin (IL)-12 monoclonal antibody exacerbates the development of EIU. The mean number of inflammatory cells ± S.E.M. was 41.6 ± 9.3 for mice treated with anti-IL-12 antibody, 17.6 ± 3.5 for mice treated with IFN-γ IL-12, and 17.7 ± 21 for control mice. Additional studies showed that intraocular administration of IL-12 inhibits the development of EIU. Similar to previous studies with anti-interferon gamma (IFN-γ) antibody, systemic

anti-IFN-γ antibody exacerbates intraocular inflammation. This may be due to decreased generation of Th-1 cells and increased generation of Th-2 cells.

(3) MDL 28,842, a potent irreversible inhibitor of S-adenosyl-L-homocysteine hydrolase was shown to inhibit the development of EAU in mice. Animals treated with MDL 28,842 at 2.5 and 5.0 mg/kg/day had significantly less disease when compared with controls ($p < 0.009$). Importantly, there was no significant weight loss in the treated animals. These data suggest that MDL 28,842 may be a useful therapeutic agent in the treatment of uveitis in humans.

(4) Topical heparin significantly inhibited the development of allergic conjunctivitis in mice. Preliminary studies revealed no ocular toxicity. These findings suggest that topical heparin may provide a well-tolerated and effective treatment for allergic conjunctivitis.

Significance to Biomedical Research and the Program of the Institute

One major mission of the NEI is to understand the mechanisms of sight- threatening eye disease so that new and effective therapies can be developed. The expression of cell-adhesion molecules appears to be a fundamental mechanism in the development of intraocular inflammation. Cytokines are also important in the pathogenesis of ocular inflammation, and certain cytokines such as IL-12 and IL-10 appear to have a regulatory role on uveitis. With this understanding, we hope to develop new anti-inflammatory therapy for ocular inflammation. The testing of these therapeutic agents in models of ocular inflammation allows the development of new therapies for patients with ocular inflammatory disease, which accounts for approximately 10 percent of the visual impairment in the United States.

Proposed Course

We plan to continue our experiments investigating the role of cell-adhesion molecules and cytokines in uveitis. In addition to using monoclonal antibodies against adhesion molecules, we are testing small molecules to block the cell adhesion molecules because these agents may be administered topically. We have started experiments studying the role of CAMs and cytokines in other inflammatory diseases, including secondary glaucoma, corneal allograft rejection, and intraocular malignancies. We are also continuing our experiments with MDL 28,842.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Anderson W, Chan C-C, Nussenblatt RB, Whitcup SM: Topical heparin inhibits compound 48/80 induced allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 35(suppl):1291, 1994.

Jang S, Li Q, Whitcup SM, Peng B, Nussenblatt RB, Chan C-C: Susceptibility to endotoxin induced uveitis varies in different murine strains. *Invest Ophthalmol Vis Sci* 35(suppl):1685, 1994.

Walton RC, Lai JC, Chanaud NP, Chan C-C, Gery I, Whitcup SM: Inhibition of experimental autoimmune uveitis by MDL 28,842. *Invest Ophthalmol Vis Sci* 35(suppl):1865, 1994.

Whitcup SM, Hayashi S, Rizzo, Lai JC, Gazzinelli R, Nussenblatt RB, Chan C-C: Systemic anti-IL-12 antibody exacerbates endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci* 35(suppl):1481, 1994.

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Chan C-C: Monoclonal antibodies against CD54 (ICAM-1) and CD11a (LFA-1) prevent and inhibit endotoxin-induced uveitis. *Exp Eye Res,* in press.

Zierhut M, Chan C-C, Duijvestijn A, Nussenblatt RB, Whitcup SM: High endothelial venules in IRBP-induced experimental autoimmune uveitis. *Invest Ophthalmol Vis Sci* 35(suppl):1807, 1994.

Patents

U.S. patent (applied for)

Contract/CRADA Reports

Allergan CRADA

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00269-04 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Ocular Toxicity of 2′,3′-Dideoxyinosine (ddI)

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Scott M. Whitcup	M.D.	Staff Medical Officer	CB, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Rafael Caruso	M.D.	Visiting Scientist	OGCS, NEI

COOPERATING UNITS (*if any*)

Pediatric Branch, National Cancer Institute (Philip A. Pizzo, M.D.); Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases (Clifford H. Lane, M.D.); Clinical Oncology Program, National Cancer Institute (Robert Yarchoan, M.D.)

LAB/BRANCH

Clinical Branch/Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0.0

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

This project, "Ocular toxicity of 2′,3′-Dideoxyinosine (ddI)", was previously listed in the Laboratory of Immunology but is now a project in the Clinical Branch. ddI is a purine analog with antiretroviral activity currently used to treat patients with the acquired immunodeficiency syndrome (AIDS), both adults and children, in clinical protocols at the NIH. The purpose of this study is to prospectively follow patients treated with ddI for the development of ocular complications secondary to drug toxicity. Ninety-five children with symptomatic (CDC class P-2) HIV infection were enrolled in a Phase I-II study to assess the safety and antiretroviral activity of ddI. More than 100 children treated with ddI have been examined, and 6 children have developed peripheral atrophy of the retinal pigment epithelium during ddI therapy. Eyes with ddI-associated retinal lesions have now been examined histologically. Microscopic examination of these lesions revealed multiple areas of retinal pigment epithelial (RPE) loss, some surrounded by areas of hypertrophy or hypopigmentation of the RPE. Partial loss of the choriocapillaris and neurosensory retina were also noted in areas of diseased RPE. Transmission electron microsocopy showed numerous membranous lamellar inclusions and cytoplasmic bodies in the RPE cells. These data show that didanosine primarily affects the RPE and that the choriocapillaris and overlying neurosensory retina are also dystrophic in areas of RPE loss. We also continue to follow a group of 75 adults treated with ddI with periodic fundus examinations and electro-oculograms. One adult previously developed retinal lesions while treated with ddI, but no additional adult patients have developed retinal lesions.

Project Description

Additional Personnel

R. Christopher Walton	M.D.	Senior Staff Fellow LI, NEI

Objectives

The goal of this study is to monitor patients treated with dideoxyinosine (ddI) for the development of ocular complications.

Methods

(1) Patients treated with ddI are given complete eye examinations every three to four months, including dilated ophthalmoscopy and fundus photography of any abnormal retinal findings. Patients treated with the higher dosages of ddI also receive periodic electro-oculograms to assess the electrophysiologic function of the retinal pigment epithelium (RPE).

(2) Eyes from one child with ddI-associated retinal lesions were studied histologically. Eyes were fixed in 10 percent buffered formalin for routine histology and electron microscopy. Sections were stained with hematoxylin and eosin, periodic acid Schiff, and Gomori's methenamine silver. Small fragments of chorioretinal tissue in the area of the lesions were embedded in an epoxy resin, and ultrathin sections were stained with uranyl acetate and lead citrate for transmission electron microscopy.

Major Findings

(1) Six children have now developed peripheral atrophy of the RPE during ddI therapy. The lesions are scalloped areas of RPE atrophy with hyperpigmented borders and occur predominantly in the midperiphery of the fundus in both eyes. These retinal lesions slowly progress if ddI therapy is continued, but central visual acuity remains unaffected. During the past year, one child developed a few retinal lesions while on ddI.

(2) No additional adults developed retinal lesions.

(3) Gross examination of the eyes revealed multiple 1 to 2 mm round lesions of RPE loss, many with areas of RPE hyperpigmentation at their periphery or centrally located. The lesions spanned from 3 mm posterior of the ora serrata to the midperiphery and extended circumferentially in each eye. Microscopic examination revealed a normal cornea, iris, ciliary body, lens, and optic nerve in both eyes. There were multiple areas of RPE loss, and some of these areas of RPE hypertrophy and/or RPE hypopigmentation were located at their margins. Chorioretinal adhesion and partial loss of the choriocapillaris and outer retina to the level of the inner nuclear layer were also noted in areas of RPE loss. Transmission electron microscopy revealed enlarged RPE cells with large, spherical melanin granules (RPE hypertrophy) and other RPE cells with a reduced number of pigment granules (RPE hypopigmentation). Degenerating RPE and neural cells were also seen. Bruch's membrane was intact, except in the areas of chorioretinal adhesion. A large number of membranous lamellar inclusions up to 20 nm in thickness and concentric membranous cytoplasmic bodies measuring up to 2 μm in diameter were clustered between the melanin granules of some RPE cells.

Significance to Biomedical Research and the Program of the Institute

It has been determined that ddI is a drug with *in vitro* and *in vivo* activity against human immunodeficiency virus infection. One of the missions of the NEI is to monitor patients for the development of ocular toxicity and assess the effect such toxicity has on vision.

Proposed Course

The detailed study of the retinal lesions associated with ddI has been completed. Although all HIV patients followed on NIH protocols will continue to be followed for the development of ocular complications, this specific project was completed on September 30, 1994.

NEI Research Program

Retinal Diseases—Photoreceptors and Pigment Epithelium

Publications

Nguyen B-Y, Shay LE, Wyvill KM, Pluda JM, Brawley O, Cohen RB, Whitcup SM, Venzon DJ, Broder S, Yarchoan R: A pilot study of sequential therapy with Zidovudine plus acyclovir, didanosine, and dideoxycytidine in patients with severe human immunodeficiency virus infection. *J Infect Dis* 168:810-817, 1993.

Whitcup SM, Dastgheib K, Nussenblatt RB, Walton RC, Pizzo PA, Chan C-C: A clinicopathologic report of the retinal lesions associated with didanosine. *Arch Ophthalmol,* in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00264-04 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cytokines and Ocular Antigens in the Eye

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	LI, NEI
	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
	Qian Li	M.D.	Visiting Fellow	LI, NEI
	Louis Kasner	M.D.	Fellow	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated and combined with project number Z01 EY 00222-08 LI.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00115-16 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cyclosporine Therapy in Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
Others:	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Scott Whitcup	M.D.	Senior Staff Fellow	LI, NEI
	Chi-Chao Chan	M.D.	Medical Officer	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cyclosporine (Cs), an endecapeptide fungal product with specific anti-T-cell characteristics, is being administered to patients with sight-threatening ocular inflammatory disease of noninfectious origin who have failed on either corticosteroid or cytotoxic agent therapy to test its efficacy in the treatment of uveitis. Within the context of these ongoing studies, the combined use of CsA and ketaconazole has been tested in a randomized masked study of a small group of patients whose uveitis was well controlled with Cs. The combination allowed a significant reduction in the dose of Cs needed to control the disease. In some instances, the dose could be reduced by as much as 90 percent. No significant increase in side effects was noted. A phase I/II randomized trial using CsA and CsG has ended. There is a definite trend showing that combined use of a Cs and low to moderate steroid doses is efficacious in preventing the progression of uveitis. An effective dose of Cs appears to be around 5 mg/kg. At this dosage, toxicity has been reduced for up to 12 months of followup. CsG was more effective than CsA in treating cystoid macular edema. Patients who remain on Cs long term continue to be followed to gain further information about renal (or other) toxicity.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Barry Grubbs	Biologist LI, NEI

Clinical Protocol Number

81-EI-33

Objectives

Cyclosporine (Cs), an endecapeptide obtained from fungi, has been shown to have specific anti-T-cell activity (*Transplant Proc* 12:234, 1980). We have reported cyclosporine's exceptional effectiveness in preventing the induction of S-antigen autoimmune uveitis in rats as well as inhibiting the disease once immunization has occurred (*J Clin Invest* 67:1228, 1981). The goal of this study is to test CsA versus CsG to test their efficacy in treating patients with bilateral sight-threatening posterior uveitis of an autoimmune nature.

Methods

Patients 18 years of age or older, of either sex (females not pregnant), who have not done well on more conventional medical therapy were admitted to this study. All patients must have a bilateral sight-threatening uveitis of noninfectious etiology that was not satisfactorily controlled by either corticosteroid or cytotoxic agent therapy. Lymphocyte cultures are prepared, and the immune cells are tested against various crude ocular extracts as well as purified human S-antigen to assess evidence of cellular immune memory, which is considered to be the *in vitro* equivalent of the anamnestic response *in vivo*. Patients chosen are treated with CsA or a new analog called CsG in a phase I/II trial to evaluate the safety and activity of CsG versus CsA. During this period, the patients' clinical and immunologic courses are closely monitored. Specific attention is given to renal function changes, a frequent side effect. Patients who need to continue CsA because of their ocular disease for more than one year may be asked to undergo renal biopsy to evaluate the reversible and irreversible components to CsA renal toxicity. Some patients who have been entered on previous CsA studies and have continued to be followed in the eye clinic will continue to be monitored for their renal function and to determine how and when cyclosporine can safely be tapered.

Major Findings

CsA has been effective in the treatment of some cases of posterior uveitis. An improvement in the inflammatory activity and visual acuity was seen in most patients treated to date. The particular responsiveness of patients with the ocular manifestations of Behçet's disease to this agent has been corroborated by a masked, randomized trial performed in Japan. The improvement in the clinical condition was supported by a concomitant improvement in electrophysiologic testing, particularly contrast sensitivity.

Patients treated with CsA had no abnormalities of natural killer cell activity before the initiation of therapy, nor was any noted afterward. CsA significantly decreased skin test responsiveness but did not alter lymphocyte proliferation or antibody production in patients. Renal toxicity has been noted in some patients on long-term therapy, necessitating the addition of systemic corticosteroids and a decrease in CsA dosage. At three months, approximately 78 percent of the patients entering this open study were considered therapeutic successes, but 62 percent were considered successes at one year.

Seventeen patients treated long term with CsA underwent renal biopsy. These biopsy specimens were read in a masked fashion by a group of renal disease specialists who compared these biopsies with those from age-matched controls. An irreversible component of CsA toxicity could be identified in the main being renal tubular atrophy accompanied by interstitial fibrosis. The majority of the individuals' biopsies had normal serum creatinine values, but a correlation could be made

between the alterations noted and previous serum creatinine elevations for some period of time. The Cs A/G trial has shown that CsG and CsA have overall equal value in treating uveitis. However, CsG was more effective in reducing cystoid macular edema than was CsA, particularly at lower dosages.

Significance to Biomedical Research and the Program of the Institute

Uveitis is one of the most frustrating problems in all of ophthalmology. Present modes of therapy for patients with severe ocular inflammatory disease are inadequate and nonspecific. CsA appears effective in treating posterior uveitis of noninfectious etiology. This is the first new agent in decades to be found useful in treating the severe form of this condition; therefore, it is important that the optimum therapeutic schedule be developed. Newer therapeutic strategies have already begun.

Proposed Course

Newer studies to look at various Cs combinations will continue.

NEI Research Program

Retinal and Diseases—Inflammatory Diseases

Publications

de Smet MD, Nussenblatt, RB: Clinical use of cyclosporine in ocular disease. *Int Ophthalmol Clin* 33(4):31-45, 1993.

Nussenblatt RB, deSmet MD, Rubin B, Freidlin V, Whitcup SM, Davis J, et al: A masked randomized, dose response study between cyclosporine A and G in the treatment of sight-threatening uveitis of noninfectious etiology. *Am J Ophthalmol* 115:583-591, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00278-03 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Oral Administration of Antigen and the Ocular Immune Response

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
Others:	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
	Susan Whitcher	M.S.	Clinical Protocol Assistant	LI, NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The effect of the oral administration of various antigens on the ocular immune response has been tested in the animal model for severe intraocular inflammatory disease, experimental autoimmune uveioretinitis, which is induced by both retinal S-antigen (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP). Oral tolerance could be induced by repeatedly feeding rats with S-Ag. A putative suppresser cell that was CD8 positive could be isolated from the spleen of such animals and transferred to other animals to induce a similar toleragenic effect. In addition, the role of the spleen was confirmed in ongoing animal experiments. A randomized masked trial to evaluate the usefulness of S-Ag feeding in patients with intraocular inflammatory diseases continues. A pilot study was performed in two patients that showed the induction of such tolerance, and these patients continue to be followed.

Project Description

Objectives

Exploring means at immunomodulation has been a major role of this laboratory. Although extensive experimentation has used various immunosuppressive agents, there has also been a major thrust in an attempt to use other modes of immunosuppression. The goal of this series of experiments, in animals as well as in humans, is to test the efficacy of oral tolerance with uveitogenic antigens in the treatment of animals induced with experimental autoimmune uveitis and in patients with bilateral sight-threatening posterior and intermediate uveitis of an autoimmune nature.

Methods

Six- to 10-week-old Lewis rats of either sex are used in these experiments. Animals are fed various antigens before and after the induction of experimental uveioretinitis. The feeding regimen includes whole molecules such as the retinal S-antigen (S-Ag) and interphotoreceptor retinoid-binding proteins as well as their fragments. In a subset of experiments, some animals will also undergo splenectomy before the initiation of these experiments, but others will receive sham procedures. We are attempting to evaluate the clinical course of their disease and corroborate the clinical observations with histopathology at various points after the initiation of the experiments. The goal is to evaluate the role of the spleen as well as the role of various fragments in the ability to induce this tolerogenic state.

In the studies to be performed with patients, those individuals who have bilateral uveitis of a noninfectious cause and who are 18 years or older of either sex will be considered for the study. Additionally, their lymphocytes must demonstrate an *in vitro* prelif-orative response to the retinal S-Ag. The patients also need to be on systemic immunosuppressive therapy, whether it be corticosteroids, cytotoxic agents, or cyclosporine. The goal of this study will be to assess, in individuals who need high amounts of immunosuppressive therapy to control their disease, whether the addition of oral feeding of retinal antigens will induce a toleragenic state.

This study will be performed in a randomized, double-masked fashion in which some patients will receive S-Ag, other patients will receive a retinal mixture containing several antigens, and still others will receive placebo. The intent is to reduce the amount of immunosuppressive therapy they are taking with the hope that a toleragenic state can be induced by the feeding of these antigens.

Major Findings

In the animal work, the spleen appears to play an important role in the induction of oral tolerance of S-Ag. In addition, the spleen is essential for adoptive transfer of tolerance by splenocytes from S-Ag fed donors. Thus, it would be logical to assume that the spleen acts as a sight for induction and/or amplification of cells with suppressive activity.

The pilot study has demonstrated that, at least in two patients, a toleragenic state could be induced with the feeding of antigen at the dosages that are planned for this study. One patient with par planitis and the other with Behçet's disease have been able to discontinue their medication completely or reduce it to exceptionally low dosages.

Significance to Biomedical Research and the Program of the Institute

Uveitis is one of the most frustrating problems in all of ophthalmology. The present modes of therapy for patients with severe ocular inflammatory disease all have limitations, particularly because of their secondary side effects. In identifying patients with an immune response to the retinal S-Ag, we will have been able to induce an immunosuppressive state without the use of pharmacologic agents. Furthermore, the induction of this tolerance would be antigen specific.

Proposed Course

The randomized study will begin shortly.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Nussenblatt RB, de Smet MD, Weiner HL, Gery I: The treatment of the ocular complications of Behçet's disease with oral tolerization, in Wechsler B, Godeau P (eds): *Sixth International Conference on Behçet's Disease.* New York, Excerpta Medica, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00184-12 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular and Immunogenetic Mechanisms in Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Rachel R. Caspi	Ph.D.	Visiting Scientist	LI, NEI
Others:	Phyllis Silver	B.S.	Biologist	LI, NEI
	Luiz Rizzo	M.D.	Visiting Associate	LI, NEI
	Gil Sartani	M.D.	Visiting Fellow	LI, NEI
	Xu Hui	Ph.D.	Visiting Fellow	LI, NEI
	Sun Bing	Ph.D.	Visiting Fellow	LI, NEI
	Chi-Chao Chan	M.D.	Medical Officer	LI, NEI

COOPERATING UNITS *(if any)*

National Institute of Child Health and Development (Lawrence M. Nelson, M.D.); Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases (Ronald L. Wilder, M.D., Ph.D.); Bone Marrow Transplantation Unit, National Cancer Institute (Frances Hakim, Ph.D.); Research and Development, Wills Eye Hospital, Philadelphia, PA (Larry A. Donoso, M.D., Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.1	5.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cellular mechanisms in ocular immunologically mediated disease are being studied in animal models of experimental autoimmune uveoretinitis (EAU). Rats and mice are immunized with retina-derived antigens or synthetic peptides, representing fragments of these antigens, to induce EAU. Susceptibility to disease induction is being evaluated in various strains of known genetic makeup, in the hope of delineating the hereditary mechanisms that predispose to uveitis. *In vivo* functional long-term T-cell lines and clones are developed from lymphoid organs of rats and mice immunized with uveitogenic ocular proteins. The functional properties and antigen receptors of these cells are being studied to develop strategies for *in vivo* targeting of the autoimmune cells. EAU in rats and mice serves as a template for the evaluation of new drugs and compounds as well as for the study and characterization of the participating cells and their factors. The goals of these studies are to identify the immunogenetic factors predisposing to uveitic disease, learn about the pathogenic mechanisms involved, characterize the immunoreactive cells and their mediators, and finally to utilize this knowledge for designing rational approaches to immunotherapy.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Robert B. Nussenblatt	M.D.	Scientific Director, NEI Chief, LI, NEI
Charles E. Egwuagu	Ph.D.	Staff Fellow, NEI
Rashid Mahdi		Biologist, LI, NEI
Igal Gery	Ph.D.	Head, Section on Experimental Immunology, NEI

Objectives

The development and study of animal models of experimental ocular autoimmune disease permits the study of cellular and genetic factors that may be generally involved in ocular autoimmunity. Experimental autoimmune uveitis (EAU) in rats and mice serves as a template for the evaluation of new drugs and compounds as well as for the study and characterization of the participating cells and their factors. Long-term maintenance of T cells *in vitro* permits the investigators to separate and selectively grow various T-cell subsets. The goals are to use the EAU model in rats and mice for the study of cellular mechanisms in ocular autoimmunity. Specifically, we are trying to delineate discrete stages in the immunopathogenic process and to devise strategies to specifically disrupt them at critical points.

One means to this end is to establish, characterize, and use retinal antigen-specific T-cell lines and clones. These lines and clones permit us to learn about cells capable of ocular immunomodulation in the positive and negative sense, to learn about migration and localization of autoimmune lymphocytes, and to study their interactions with other lymphoid and nonlymphoid cells in eliciting effector mechanisms. Finally, we use the EAU model for the study of hereditary mechanisms controlling genetic susceptibility and resistance to ocular autoimmune disease. The study and understanding of these parameters will help not only in the development of new therapies but also possibly in the prevention of ocular disease.

Methods

Rats and mice of various strains are immunized with purified S-antigen (S-Ag) or with interphotoreceptor retinoid-binding protein (IRBP) in complete Freund's adjuvant or with various pathogenic peptides derived from these proteins that are synthesized in the laboratory. After the development of disease, eyes are processed for histopathology and examined for disease, and lymphoid cells from the blood, lymph nodes, or eyes are taken. Cells thus obtained are placed in culture either with mitogen or with the retinal antigen with which the donor animal was immunized. Responses of the immune cells are studied.

Cells are also expanded in culture and used to transfer EAU to nonimmune animals to study the cell population responsible for disease induction. Similar methods are used to study cells responsible for disease suppression. Long-term cell lines are developed and in some cases are cloned. These cell lines and clones are then tested for functional characteristics such as the ability to induce or suppress ocular disease, production of soluble mediators, expression of various cell-surface molecules, response to therapeutic agents, and interactions with other cells in culture.

Major Findings

The possible correlation between the pathogenicity of autoimmune T cells and their lymphokine production, expression of functional adhesion molecules and expression of some surface antigens, was examined in the Lewis rat EAU model. We used four retinal antigen-specific Lewis rat T-cell lines and sublines: one specific to the major pathogenic epitope of the human retinal soluble antigen (S-Ag; residues 337-356), and three specific to the major pathogenic epitope of the bovine IRBP (residues 1177-1191). The lines have different degrees of uveitogenicity, from highly pathogenic to nonpathogenic. All four T-cell lines produced roughly equivalent amounts of interferon

gamma (IFN-γ), lymphotoxin/tumor necrosis factor (TNFα/β), interleukin (IL)-3, IL-6, and transforming growth factor beta (TGF-β). IL-4 activity could not be detected. The lines also expressed similar levels of functional adhesion molecules, as measured by binding to cultured rat aorta endothelial cells. The nonpathogenic subline, however, was the lowest responder to antigenic stimulation with respect to proliferation and IL-2 production. Examination of cell-surface antigens showed that in contrast with the other lines, the majority of cells in the nonpathogenic subline lacked detectable expression of CD-4. No difference was found in the level of expression of the IL-2 receptor and T-cell antigen receptor between the four lines. Because CD-4 is the restricting element in these lines, reduced CD-4 expression in the nonpathogenic subline may at least partially explain its poor response *in vitro* to antigenic stimulation.

All three attributes could be connected to lack of pathogenicity of this line *in vivo.* These results support the contention that class II-restricted recognition of autoantigen within the neuroretina by uveitogenic T lymphocytes must occur as an initial step in the pathogenesis of EAU. A defect in this step will preclude pathogenesis, regardless of some other functional attributes possessed by effector T cells such as production of inflammatory lymphokines and expression of adhesion molecules.

We have also studied the expression of cytokine genes within the eye in murine EAU. We have previously described several T-cell lines specific to the retinal protein IRBP or to its peptides that can induce EAU on adoptive transfer into naive mice. We have also shown that such cell lines elaborate an unrestricted cytokine profile *in vitro.*

The purpose of this study was to investigate what set of cytokines was being expressed by these cells *in vivo* within the target organ. C57BL/6 athymic mice and B10.A euthymic mice were injected intravenously with histocompatible uveitogenic T cells. Control animals were immunized with an established uveitogenic regimen of IRBP. The eyes were harvested 11 to 21 days later after perfusing the animals with 10 mL of PBS/heparin (to ensure that the cytokines detected did not originate from passenger lymphocytes passing through the retinal vessels). One eye was sent for histological examination, and the other eye was processed for ribonucleic acid (RNA) extraction. The RNA was reverse-transcribed, and the resulting complementary deoxyribonucleic acid was amplified using primers for several cytokines of interest. The results showed that although the uveitogenic T-cell lines and clones had an unrestricted cytokine profile *in vitro,* predominantly Th-1-type cytokine messenger ribonucleic acid (mRNA) (IL-2, IFN-γ, and TNF-α) were present in eyes of mice that had EAU induced with those cells. Eyes of actively immunized animals showed a less restricted lymphokine profile, with most eyes having detectable mRNA for IL-2, IFN-γ, and IL-4. Eyes of control animals that had neither been immunized nor adoptively transferred with T cells were negative for all cytokines tested. These results suggest that IL-2, IFN-γ, and TNF-α are involved in murine EAU. Taken together with a lack of IL-4 mRNA in eyes of mice that had EAU induced by adoptive transfer, the data argue that predominantly Th-1 type cells were present in the uveitic eyes. Because adoptive EAU was induced with T-cell lines having an unrestricted cytokine profile (representing a Th-0 or a mixed Th-1+Th-2 population), this might imply that either a differentiation or a selection event occurred during development of EAU.

In the mouse model of EAU, we have identified a major pathogenic epitope in the IRBP molecule that is recognized by mice of the H-2r haplotype. Overlapping 20-amino acid peptides, spanning the entire human IRBP molecule, were synthesized and used to immunize C57BL/10 (H-2b), B10.BR (H-2k), and B10.R111 (H-2r) mice. Bovine IRBP was used as a positive control. EAU was examined by histopathology 28 days after immunization. *In vivo* and *in vitro* immunological responses were assessed by delayed type hypersensitivity (DTH) and lymphocyte proliferation, respectively.

Peptide 161-180, spanning the sequence SGIPYVISYLHPGSTVSHVD, was found to be highly pathogenic for B10.RIII mice but not for the other strains. EAU occurred in 20/20 B10.RIII mice after immunization with 50 μg of peptide. The average disease score was 2.7 as compared with 3.2 for IRBP-immunized mice. A dose-response curve showed that peptide 161-180 remained maximally pathogenic at 25 μg, but incidence and scores were reduced at 10 μg. The truncated 14-mer 165-178 was also highly pathogenic (100-200 μg/mouse), suggesting that it contained the pathogenic epitope. Mice immunized with the peptide, or with whole IRBP, had positive DTH and lymphocyte responses *in vitro* to the immunizing as well as to the reciprocal antigen. These results indicate that peptide 161-180 appears to contain an epitope that is pathogenic to mice of the H-2r, but not H-2b or the H-2k haplotypes. High incidence and high severity scores as well as immunological crossrecognition between the peptide and IRBP *in vivo* and *in vitro* suggest that this peptide contains a major pathogenic epitope.

In another study, the compound suramin was evaluated for its efficacy to prevent EAU. Suramin has been in clinical use for treatment of parasitic diseases and some types of cancer and is known to have immunosuppressive properties. EAU was induced in B10.A mice by immunization with the whole IRBP protein and in Lewis rats by immunization with peptide 35 of S-Ag or by adoptive transfer of a T-cell line specific to this peptide (SP-35 line).

Actively immunized animals were treated with suramin (30-100 mg/kg, 1.P.) to cover either the afferent or the efferent stage of EAU (days 0 and seven or days seven and 14, respectively). Adoptively transferred animals (considered to represent efferent-stage disease) were treated on day minus one. Control animals were injected with phosphate-buffered saline at the corresponding times. EAU was assessed by clinical evaluation and by histopathology performed approximately one week after onset. Immunological responses were assessed by DTH and lymphocyte proliferation to the immunizing antigen. The results revealed that treatment of B10.A mice with 100 mg/kg of suramin completely prevented disease when given during the afferent stage and ameliorated disease when given during the efferent stage of EAU. The same dose and regimen were somewhat less effective in preventing EAU in Lewis rats: Afferent treatment lowered the incidence and ameliorated disease scores by approximately 50 percent, whereas efferent treatment (of either immunized or adoptively transferred rats) had little or no effect on disease. Effect on DTH responses and lymphocyte proliferation roughly paralleled the effect on EAU. Thus, afferent treatment with suramin suppressed EAU and immunological responses, whereas treatment during the efferent stage was less effective, suggesting interference with antigen priming. The response to treatment may in part be dependent on the species in that mice responded to the same treatment better than rats. To our knowledge, this is the first report of the successful use of suramin for treatment of autoimmunity.

Another approach to suppressing ocular autoimmunity was through induction of oral tolerance. It was previously shown that EAU in rats can be suppressed by feeding of S-Ag. We wished to: (1) test whether a similar phenomenon exists in mice and (2) evaluate the feasibility of potentiating it by immunomanipulation. For this purpose, EAU-susceptible B10.A mice were fed IRBP or control solution using various regimens and were subsequently challenged with a uveitogenic regimen of IRBP. EAU was assessed by histopathology 21 days after immunization. Immunological responses measured included DTH, lymphocyte proliferation, and cytokine production.

The results indicated that three feedings of 0.2 mg IRBP every other day before immunization did not protect mice against EAU, whereas a similar regimen of five feedings of 0.2 mg IRBP every other day was protective. However, supplementing the nonprotective 3x regimen with one intraperitoneal administration of 400 units of recombinant human IL-2

on the day of immunization resulted in disease suppression that was equal to that of the protective 5x regimen ($p \leq 0.02$ compared with unfed controls). Analysis of cytokines produced by Peyer's Patch cells of fed mice showed a large increase in production of TGFb, IL-4, and IL-10 in the 3x-fed and IL-2-treated animals compared with animals given the nonprotective 3x (no IL-2) feeding regimen and animals given the protective 5x feeding regimen. We propose that the IL-2 treatment enhances protection from EAU by stimulating regulatory cells that produce TGF-β, IL-4, and IL-10. Furthermore, the differences in lymphokine production patterns among the experimental groups suggest that the mechanism of protection induced by the 3x + IL-2 regimen may differ from that induced by the 5x regimen. It is conceivable that in the former case, protection from EAU was achieved by active suppression of the uveitogenic effector cells, whereas a mechanism of deletion or anergy might predominate in the latter.

Significance to Biomedical Research and the Program of the Institute

It has become increasingly clear that the cellular mechanisms and possibly the genetic mechanisms observed in animal models of uveitis reflect the mechanisms that operate in ocular immune-mediated disease in humans. The identification and characterization of the cells involved in ocular autoimmunity, and of their functions, will provide new understanding of inflammatory ocular diseases. Successful immunomodulation of EAU in animal models has thus far usually served as a good predictor of the clinical success of a given therapeutic modality. The continued study of basic mechanisms involved in the immunopathogenesis of uveitis in animal models will aid in the development of novel immunotherapeutic approaches for the control of uveitis in humans.

Proposed Course

This project will continue so that more information about the basic mechanisms in experimental uveitis may be obtained.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Caspi RR, Chan C-C, Grubbs BG, Silver PB, Wiggert B, Heremans H: Interferon-gamma at the systemic level protects mice against experimental autoimmune uveoretinitis. *Reg Immunol,* in press.

Caspi RR, Chan C-C, Fujino Y, Najafian F, Grover S, Silver PB, Hansen CT, Wilder RL: Recruitment of naive T cells plays a pivotal role in experimental autoimmune uveoretinitis (EAU). *Reg Immunol,* in press.

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Kozhich AT, Kawano Y, Egwuagu CE, Caspi RR, Berzofsky JA, Gery I: A pathogenic autoimmune process targeted at a surrogate epitope. *J Exp Med* 180:133-140, 1994.

Mahdi RM, Caspi RR, Kozhich AT, Kozhich OA, Silver PB, Nussenblatt RB, Egwuagu CE: Cytokine mRNA expression following adoptive transfer of uveitogenic T cells into athymic and euthymic Lewis rats. *Invest Ophthalmol Vis Sci* 35(4):1561, 1994.

Maity R, Caspi RR, Nelson LM: Neonatal thymectomy causes persistence into adulthood of the neonatal capacity for increased IL-4 production. *FASEB J* 8:A483, 1994.

Miller-Rivero NE, Rizzo LV, Chan C-C, Wiggert B, Nussenblatt RB, Caspi RR: Suppression of IRBP-induced EAU in mice by feeding IRBP, and its potentiation by interleukin-2. *Invest Ophthalmol Vis Sci* 35(4):1865, 1994.

Prendergast RA, Coskuncan NM, Lutty GA, McLeod DS, Caspi RR: Induction of adoptive T cell-mediated EAU: Temporal appearance of specific and control activated cells in retinal tissue. *Invest Ophthalmol Vis Sci* 35(4):1561, 1994.

Prendergast RA, Coskuncan NM, MacLeod DS, Lutty GA, Caspi RR: T cell traffic and the pathogenesis of experimental autoimmune uveoretinitis, in: *Advances in Ocular Immunology* (Proceedings of the Sixth International Symposium on the Immunology and Immunopathology of the Eye, Bethesda, MD, 1994). Amsterdam, Elsevier Science Publishers B.V., International Congress Series, 1994, pp 59-62.

Rizzo LV, Miller-Rivero NE, Chan C-C, Wiggert B, Nussenblatt RB, Caspi RR: Interleukin-2 treatment potentiates induction of oral tolerance. *FASEB J* 8:A476, 1994.

Rizzo LV, Miller-Rivero NE, Chan C-C, Wiggert B, Nussenblatt RB, Caspi RR: Effect of interleukin-2 on the induction of oral tolerance in experimental autoimmune uveoretinitis, in: *Advances in Ocular Immunology* (Proceedings of the Sixth International Symposium on the Immunology and Immunopathology of the Eye, Bethesda, MD, 1994). Amsterdam, Elsevier Science Publishers B.V., International Congress Series, 1994, pp 221-224.

Rizzo LV, Miller-Rivero NE, Chan C-C, Wiggert B, Nussenblatt RB, Caspi RR: Interleukin-2 treatment potentiates induction of oral tolerance in a murine model of autoimmunity. *J Clin Invest* 94:1668-1672, 1994.

Rizzo LV, Silver PB, Gazzinelli RT, Chan C-C, Wiggert B, Caspi RR: Expression of cytokine genes within the eye in murine EAU. *Invest Ophthalmol Vis Sci* 35(4):1862, 1994.

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Savion S, Oddo S, Grover S, Caspi RR: Uveitogenic T lymphocytes in the rat: Pathogenicity vs. lymphokine production, adhesion molecules and surface antigen expression. *J Neuroimmunol,* in press.

Silver PB, Rizzo LV, Chan C-C, Donoso LA, Wiggert B, Caspi RR: Identification of a major pathogenic epitope in the IRBP molecule recognized by mice of the H-2r haplotype. *Invest Ophthalmol Vis Sci* 35(4):2061, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00266-05 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Immune Responses to Retinal Specific Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
Others:	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	François Roberge	M.D.	Visiting Scientist	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0.0

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [x] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

One of the characteristics of S-antigen (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP), which are retinal-specific antigens, is the ability to induce an intense autoimmune inflammation in the eyes of experimental animals when injected in the presence of an adjuvant. This disease, called experimental autoimmune uveitis (EAU), is critically dependent on T cells and antigen processing by appropriate antigen-presenting cells (APCs). Antigen processing, which occurs within the endocytic vesicles of the APCs, results in the production of small polypeptide subunits. These small polypeptides must then be protected from further degradation and transported to the cell surface where the interaction with the T cell takes place.

In FY 1994 we further characterized the intracellular protein first identified in FY 1993. We confirmed that the protein does belong to the heat shock family of proteins by partial amino acid sequencing of the 70kD peak. We also identified the peak at 40kD as being actin. Testing with different peptide moieties has shown that binding of immunogenic peptides by hsp70 is a selective process. Certain peptides bind well to hsp70, although other peptides have no affinity for hsp70. The process is also selective in that it requires ATP in order to release the bound peptide. We have also found increased levels of hsp antibody in the serum of patients with Behçet's disease. The increase serum levels corresponded to periods of ocular inflammatory activity in the absence of any evidence of active systemic disease.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Sumeet Mainigi		Biologist, LI, NEI
Sakpana Rengerajan	Ph.D.	Biolgist, LRCMB, NEI
Gerald J. Chader	Ph.D.	Chief, LRCMB, NEI
Barbara Wiggert	Ph.D.	Head, Section on Biochemistry, LRCMB, NEI

Clinical Protocol Numbers

84-EI-214
79-EI-49

Objectives

In fiscal year (FY) 1994, we further characterized the intracellular protein first identified in FY 1993. We confirmed that the protein does belong to the heat shock family of proteins by partial amino acid sequencing of the 70kD peak. We also identified the peak at 40kD as being actin. Testing with different peptide moieties has shown that binding of immunogenic peptides by heat shock proteins (HSP) 70 is a selective process. Certain peptides bind well to HSP 70, but other peptides have no affinity for HSP 70. The process is also selective in that it requires adenosine triphosphate to release the bound peptide. We have also found increased levels of HSP antibody in the serum of patients with Behçet's disease. The increased serum levels corresponded to periods of ocular inflammatory activity in the absence of any evidence of active systemic disease.

Methods

Using B-cell lysates from Epstein-Barr virus (EBV)-transformed cells and from B-cell isolates of rat spleens, the HSP 70 capable of binding to interphotoreceptor retinoid-binding progression (IRBP) fragment 1169-1191 was isolated and further characterized by amino acid sequencing. Isolation was carried out on an activated Sepharose 4b column to which an appropriate peptide was linked. Several experiments were carried out to determine the affinity of various peptide substitutes to intracellular HSP 70. Human serum was tested in a standard enzyme-linked immunosorbent assay using a commercial HSP 70 antigen.

Major Findings

We have determined that the 70 kD binding protein belongs to the heat shock family of proteins and that it is a new member of the HSP 70 family of proteins because part of its sequence has only 40 percent homology with other HSP members. The 40 kD protein is actin. The exact role of actin in antigen presentation is still unknown. One simple explanation is that actin binds through a simple bystander phenomenon because the protein is isolated from a cell lysate. However, monomeric actin may well play a more active role in antigen presentation, particularly of cytosolic proteins. Further studies will be necessary to further elucidate its role.

Using cell lysates of EVB-transformed cells, we were able to show that the binding of HSP 70 to various peptide fragments is dependent on the peptide sequence. Substitution of certain nonpolar amino acids by charged molecules changes the binding characteristics. Differences were also noted between patients with Behçet's disease and normal individuals in terms of binding to various residues or analogs of IRBP. Of particular interest was the finding that serum levels of HSP 70 antibodies varied with the level of ocular inflammation. Patients had levels of HSP 708 antibodies that were above two standard deviations of controls, the level of HSP 70 antibodies rose significantly in patients with ocular inflammatory episodes. This is a unique finding in ocular inflammatory disease, where it is rare to find a systemic marker of inflammatory activity in the eye.

Proposed Course

In the coming year, the main emphasis will be on further elucidating the role of hsps in antigen presentation by studying the effect of

cellular stress on antigen presentation in cell lines and clones. In addition, we will attempt to produce a specific polyclonal and monoclonal antibody to the HSP 708 that was isolated in the course of these studies. Once the antibody has been generated, we will look at several more patient populations to further define the role of hsp in uveitis.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

de Smet MD, Bitar G, Roberge FG, Gery I, Nussenblatt RB: Human S-antigen: Presence of multiple immunogenic and immunopathogenic sites in the Lewis rat. *J Autoimmun* 6:587-599, 1993.

de Smet MD, Rengerajan K, Chader GJ, Wiggert B: Characterization of B cell proteins binding specifically to uveitopathogenic peptide 1169-1191 of bovine IRBP. *Invest Ophthalmol Vis Sci* 35(suppl):1865, 1994.

Mainigi S, Rengarajan K, Wiggert B, Chader GJ, Nussenblatt RB, de Smet MD: Elevation of serum antibody levels to heat shock protein 70 during ocular inflammatory episodes in Behçet's patients. *Invest Ophthalmol Vis Sci* 35(suppl):2098, 1994.

Rengarajan K, de Smet MD, Chader GJ, Wiggert B: Affinity of HSP70 from EBV transformed human B cells for bovine and human IRBP peptides. *Invest Ophthalmol Vis Sci* 35(suppl):1864, 1994.

Rengarajan K, de Smet MD, Chader GJ, Wiggert B: Identification of heat shock protein binding to an immunodominant uveitopathogenic peptide of IRBP. *Curr Eye Res* 13:298-296, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00276-03 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Surgical Management of Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
Others:	François Roberge	M.D.	Visiting Scientist	LI, NEI
	Margaret Cheung	M.D.	Senior Staff Fellow	LI, NEI
	David Parks	M.D.	Senior Staff Fellow	LI, NEI

COOPERATING UNITS *(if any)*

Clinical Oncology Program, Medicine Branch, National Cancer Institute (Robert Wittes, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [X] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

THIS PROJECT IS INACTIVE.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00218-09 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ocular Manifestations of the Acquired Immune Deficiency Syndrome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Scott Whitcup	M.D.	Assistant Clinical Director	CB, NEI
	Margaret Cheung	M.D.	Senior Staff Fellow	LI, NEI
	David Parks	M.D.	Senior Staff Fellow	LI, NEI
	François Roberge	M.D.	Visiting Scientist	LI, NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI

COOPERATING UNITS *(if any)*

Department of Critical Care Medicine, Clinical Center (Henry Masur, M.D.); Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases (H. Clifford Lane, M.D.); Pediatric Branch, National Cancer Institute (Phil A. Pizzo, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [X] (b) Human tissues [ ] (c) Neither

[X] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients suffering from AIDS (acquired immunodeficiency syndrome) are at risk of developing significant ocular problems, either as a result of HIV (human immune deficiency virus) itself or as a result of opportunistic infection. Some of these problems can lead to blindness if left untreated. Among the many pathogens that can lead to blindness, cytomegalovirus (CMV) is by far the most common.

In FY 1994, we have evaluated the effectiveness of an intraocular delivery device in preventing the spread of CMV retinitis. We have now nearly completed recruitment for this study. Although no analysis has yet been done of the data, no significant adverse reaction has been noted, and in all cases progression of CMV retinitis has been prevented.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Susan Mellow	RN	Nurse Specialist CB, NEI

Clinical Protocol Number

90-EI-208

Objectives

Patients suffering from AIDS (acquired immunodeficiency syndrome) are at risk of developing significant ocular problems, either as a result of human immunodeficiency virus itself or as a result of opportunitistic infection. Some of these problems can lead to blindness if left untreated. Among the many pathogens that can lead to blindness, cytomegalovirus (CMV) is by far the most common.

In fiscal year (FY) 1994, we have evaluated the effectiveness of an intraocular delivery device in preventing the spread of CMV retinitis. We have nearly completed recruitment for this study. Although no data analysis has yet been done, no significant adverse reaction has been noted, and in all cases progression of CMV retinitis has been prevented.

Methods

This project entails the clinical evaluation, diagnosis, and treatment of retinitis in AIDS patients. It also involves the development of novel methods of therapy for the various forms of retinitis observed. Study of pathologic tissue also is used to better understand the nature of the infectious processes.

Major Findings

In the past year, our major effort has centered on the treatment of CMV retinitis by using a slow release intraocular device. CMV retinitis is a major vision-threatening infection found in patients with advanced stages of AIDS. Current therapeutic modalities commit patients to life-long intravenous (IV) therapy with anti-CMV drugs. These drugs require close monitoring because of their systemic toxicity. They also require the placement of a permanent IV access and hence place the patient at risk for both local and systemic infection. Recurrence of disease also tends to occur in the majority of patients. An intraocular, slow-release device avoids these side effects by providing continuous antiviral therapy above the minimum inhibitory concentration for up to eight months. Recruitment of patients for the study that was started in FY 1993 is now completed. This study compares patients in whom an intraocular device is placed immediately with patients in whom placement is differed until the CMV retinitis has progressed by 750 μm. Although recruitment has been completed, some patients are still in the active phase of the study. No serious side effects have been encountered, and good control of the CMV retinitis has been achieved. Full analysis of the study parameters will be undertaken shortly. Particular attention will be given to the rate of bilateralization and to the survival of patients treated with the intraocular device.

Despite treatment, CMV retinitis has a tendency to recur. With larger areas of retinal involvement, the risk of CMV detachment increases considerably, particularly in patients with peripheral involvement where vitro-retinal traction is greatest. Up to 30 percent of patients with CMV retinitis will develop a retinal detachment. Repair of detachments requires the use of a variety of vitro-retinal techniques from classical retinopexy to the use of more advanced vitreal-retinal approaches, including the use of silicone oil. The best time for intervention still remains to be determined. In FY 1994, we have begun to explore the various surgical approaches that are available for the repair of retinal detachments in patients with CMV retinitis. For patients with detachments involving noninfected retina, standard scleral buckling procedures appear to be adequate. If a retinal detachment develops in involved retina, scleral buckling with silicone oil appears to be the most effective

approach. Many patients, however, do not recover their full central visual acuity. The cause of this decrease is not known and is being investigated.

Significance to Biomedical Research and the Program of the Institute

The AIDS epidemic is a major public health concern. CMV retinitis remains the number one cause of blindness among patients infected with the AIDS virus. Early diagnosis is important because all drugs currently available are only virostatic and not virocidal; thus, some progression of the lesion is seen in more than 50 percent of patients, despite anti-CMV therapy. No therapeutic modalities that are cost effective and that reduce the incidence of progression are necessary.

Management of the complications of CMV retinitis, particularly after recurrence and progression of the disease will be an ever-increasing challenge as patients survive longer. Of these, the threat of retinal detachment is a major concern. The means of prophylaxis and therapy need to be developed.

Proposed Course

In the coming fiscal year, we are planning to evaluate the slow-release device further as well as the means of preventing second eye involvement in patients who are treated with the device. We also will study further means of treating patients with viral-related retinal detachments and means of preventing visual loss at the time of surgery.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

de Smet MD, Nussenblatt RB: Ocular manifestations of HIV in the pediatric population, in Pizzo PA, Wilert CM (eds): *Pediatric AIDS: The Challenge of HIV Infection in Infants, Children, and Adolescents.* Baltimore, Williams & Wilkins, 1994 pp. 457-466.

Maturi RK, Nussenblatt RB, de Smet MD: Prevalence of tear hyposecretion and vitamin A deficiency in patients with AIDS. *Invest Ophthalmol Vis Sci* 35(4)(suppl):1308, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00232-09 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interferon System in Cellular Function and Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI
Others:	Caroline Percopo	M.S.	Biologist	LI, NEI
	Chandrasekharam Nagineni	Ph.D.	Visiting Scientist	LI, NEI

COOPERATING UNITS *(if any)*

Department of Pathology, The George Washington University Medical Center (Barbara Detrick, Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunology and Virology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cytokines, such as interferon gamma (IFN-γ) and interleukin (IL)-2, are a group of specialized hormone-like proteins that exert profound influences on cellular development and on a variety of cellular functions. This project has concentrated on studying the ways in which cytokines interact with cells of the immune system and with cells in the ocular microenvironment. We have shown that IFN-γ and IL-2 are found within the inflamed eye in association with T-cell infiltration and major histocompatibility complex (MHC) class II antigen expression on infiltrating cells and on retinal pigment epithelial (RPE) cells. Furthermore, IFN-γ-activated RPE cells can process and present antigens to helper T lymphocytes.

Experimentally we demonstrated that isolated human RPE cells can be induced to produce another lymphokine, IL-6, and soluble intercellular adhesion molecule-1 (ICAM-1) following incubation with IFN-γ. IL-6 is a potent inflammatory cytokine capable of enhancing antibody production and cytotoxic T-cell activities. ICAM-1 is an adhesion molecule that mediates cell adhesion, an essential component for several immunologic functions. These studies indicate that cytokine-mediated activation of RPE cells may be a basic component of ocular immunity and an important aspect of RPE cell transplantation.

These observations indicate that IFN-γ induces MHC class I, class II antigen and ICAM-1 expression and IL-6 secretion by RPE cells. All of these factors may serve as a local amplification system in autoimmune and inflammatory eye disease. A better understanding of the role of cytokines in the mechanisms involved in the development of autoimmunity and inflammation may be beneficial in developing treatments for these diseases.

Project Description

Objectives

This project is designed to determine the bioregulatory actions of interferon (IFN) and other cytokines and to evaluate their regulatory actions in the pathogenesis of disease.

Methods

We assayed human IFN using inhibition of vesicular stomatitis virus plaque formation in human amnion or WISH cells. IFNs were characterized by neutralization of antiviral activity with monoclonal anti-IFN immunoglobulin. Interleukin (IL)-2 biological activity was assayed by induction of proliferation of CTL cells. Soluble intercellular adhesion molecule 1 (ICAM-1) and IL-6 activity was assayed by an enzyme-linked immunosorbent assay (ELISA) and immunoblot assays. ICAM-1 and IL-6 messenger ribonucleic acid (mRNA) were evaluated by Northern blot assays. Analytical flow cytometry was used to quantitate retinal proteins. Gene transcription techniques such as Northern blot analysis and nuclear runoff transcription assays are being used to evaluate interferon gamma (IFN-γ) modulation of retinal proteins.

Major Findings

Numerous studies indicate that a variety of autoimmune diseases are associated with the IFN-γ-induced tissue-specific expression of major histocompatibility complex (MHC) class II molecules. During the past five years, we have identified various steps that may be involved in ocular immunopathologic mechanisms. In these studies of retinal degenerations and autoimmune diseases, we showed that a critical regulatory cell in the retina, the retinal pigment epithelial (RPE) cell, is capable of expressing MHC class II determinants. We can also detect IFN-γ, *in situ*, in retinas from patients with inflammatory eye diseases as well as MHC class II positive RPE cells. In addition, freshly isolated human RPE cells can express these determinants following treatment with IFN-γ. In animal model systems, we found that inoculation of recombinant IFN-γ induces Ia expression of ocular cells, and treatment with anti-Ia antibodies can eliminate or inhibit experimental autoimmune uveitis. Recently, we showed that the RPE cell may be playing an important role in ocular immunity, acting as a resident antigen presenting cell in the retina.

Also, we found that the RPE cell is capable of producing the cytokine, IL-6. During the past year, we have evaluated ICAM-1 production by cytokine-activated RPE cells. RPE cell cultures were established from human donor eyes. Stimulation of RPE cells with IL-1α, IL-β, tumor necrosis factor alpha (TNF-α), and IFN-γ resulted in the production and release of ICAM-1. In addition, there was a concomitant increase in the RPE cell surface expression of ICAM-1 and the production of ICAM-1 mRNA. In contrast, IL-6 and lipopolysaccharide were not capable of inducing ICAM-1 secretion by RPE cells. Taken together, these studies indicate that the proinflammatory cytokine such as IL-1, TNF, and IFN-γ can activate RPE cells to release both IL-6 and soluble ICAM-1. These studies further substantiate the concept that cytokine-mediated activation of RPE cells may be a basic component of ocular immunity and may have major immunological consequences for RPE cell transplantation studies.

Significance to Biomedical Research and the Program of the Institute

The studies described herein highlighted the fact that the release of cytokines such as IFN-γ, within the ocular microenvironment and the subsequent induction of cytokines and MHC class I and II antigen expression on resident and infiltrating cells may be critical elements in a cascading effect that leads to ocular cell destruction. The cell within the retina that may play a critical role in autoimmune uveitis is the RPE cell. IFN-γ induced activation of RPE cells may participate in autoimmune disease in the ocular microenvironment.

Cytokines produced and localized in the eye may play a critical role in normal physiology, pathogenic mechanisms, and therapeutic approaches. Because the RPE cell is a pivotal regulatory cell in the retina, an understanding of how cytokines interact with this cell will shed light on avenues for therapeutic intervention in pathogenic states and transplantation.

Proposed Course

We plan to continue our evaluation of the role of cytokines in autoimmunity and inflammation. We are now developing systems in rat models to monitor directly the effects of altering cytokine production on inflammatory eye diseases. Moreover, we will continue to characterize the antigen-presenting ability of the RPE cell to a variety of antigens and viruses.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Detrick B, Hooks JJ: Cytokines and effector molecules in human immunology, in Leffell MS, Bias WB, Donnenberg AD, (eds): *CRC Handbook of Human Immunology.* Boca Raton, Florida, CRC Press Inc., in press.

Nagineni CN, Detrick B, Hooks JJ: Synergistic effects of gamma interferon on inflammatory mediators that induce interleukin-6 gene expression and secretion by human retinal pigment epithelial cells. *Clin Diag Lab Immunol* 1:569-577, 1994.

Nagineni CN, Detrick B, Hooks JJ: Human RPE cells secrete cytokines in response to inflammatory mediators, in *Proceeding of the Sixth International Symposium on the Immunology and Immunopathology of the Eye.* Amsterdam, Elsevier Science Publishers, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00233-09 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Bioregulatory Aspects of the Retinal Pigment Epithelial Cell

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI
Others:	Chandrasekharam Nagineni	Ph.D.	Visiting Scientist	LI, NEI
	Caroline Percopo	M.S.	Biologist	LI, NEI
	T. Michael Redmond	Ph.D.	Research Biologist	LRCMB, NEI
	R. Krishnan Kutty	Ph.D.	Senior Staff Fellow	LRCMB, NEI
	David Parks	M.D.	Senior Staff Fellow	LI, NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	LI, NEI

COOPERATING UNITS *(if any)*

Hôpital St. Louis, Paris, France (Lawrence Boumsell, M.D.); University of Nice, France (Alain Bernard, M.D.); National Institute of Dental Research (Reuben Siraganian, M.D.); The Johns Hopkins University (Stanley A. Vinores, Ph.D.; Peter Campochiaro, M.D.); Department of Pathology, The George Washington University Medical Center (Barbara Detrick, Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunology and Virology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.9	0.5	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects x (b) Human tissues (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The retinal pigment epithelial (RPE) cell plays a basic role in maintaining the structural and physiological integrity of the neural retina. We have isolated and propagated RPE cells *in vitro* and have developed monoclonal antibodies directed against human RPE cells. The RPE epitope is a 67 kDa protein that is closely associated with the microsomal membrane. A complementary deoxyribonucleic acid (cDNA) clone has been isolated that codes for a protein that does not match any other sequences in the databases. We are using these techniques and reagents to evaluate molecular, biochemical, and biological properties of the RPE cells.

We have propagated human RPE cells *in vitro* and evaluated their ability to respond to cytokine activation. Transforming growth factor - β (TGF-β) is a potent cytokine that modifies a variety of cellular functions. This cytokine has been identified within the retina. We found that TGF-β induces gene expression and production of Heme oxygenase (HO-1). Since HO-1 is a protective agent against oxidative damage in an O_2 rich environment, RPE production of this protein may protect the retina against oxidative damage.

Studies are also in progress to propagate and transplant RPE cells in various animals. We have established a graft rejection model by transplanting human RPE cells into the rat retina. These studies demonstrate that both cellular and humoral aspects of the immune response are activated to reject RPE cell transplants. These studies provide the framework to evaluate cytokines and immune reactivity in RPE cell transplantation.

Project Description

Objectives

The aim of this project is to evaluate the molecular, biochemical, and varied biologic properties of retinal pigment epithelial (RPE) cells in normal and disease states. Moreover, we are evaluating RPE cell transplantation.

Methods

RPE cells are isolated and propagated *in vitro*. Monoclonal antibodies were generated in mice by fusing mouse spleen cells with myeloma cells. Antibodies to RPE cells are evaluated by immunoperoxidase assays and Western blot assays. The effect of drugs and cytokines are evaluated by cell viability and proliferation assays and nuclear transcription runoff assays.

Major Findings

(1) Cytokine Activation of RPE Cells. Transforming growth factor beta (TGF-β) is a potent cytokine that modifies a variety of cellular functions. This cytokine has been identified within the retina. We found that TGF-β induces messenger ribonucleic acid (mRNA) for heme oxygenase-1 (HO-1) and induces HO-1 protein expression in human RPE cells. Because HO-1 is a protective agent against oxidative damage in an oxygen rich environment, RPE production of this protein may provide downregulation of oxidative damage.

(2) *RPE cell transplantation.* Recent studies indicate that RPE cell transplantation may be beneficial in restoration of retinal architecture in selected retinal degenerations. It is essential to develop methods for large-scale preparations of RPE cell cultures for somatic cell genetic engineering manipulations. We are in the process of evaluating various parameters for human and rat RPE cell culture and transplantation. Preliminary studies show that we can successfully transplant human RPE cells into the rat retina. We have used this xenogeneic RPE transplant in the rat as a graft rejection model. We demonstrated that the transfer of cultured adult human RPE cells to the Lewis rat subretinal space elicits a graft rejection peaking at seven days posttransplantation. Infiltrating cells consisted of phagocytic CD-11c/CD-18 cells as well as CD-4 and CD-8 positive cells. Systemic antibodies to the human RPE cells developed in the rats by seven to 21 days. These data indicate that both cellular and humoral aspects of the immune response are activated in this graft rejection model.

Significance to Biomedical Research and the Program of the Institute

The monoclonal antibodies developed in this study are the first directed solely at the RPE cell. These antibodies are potentially useful in identification of RPE cells *in situ* and *in vitro*. These antibodies, which can be used to monitor RPE cellular functions, may be used in providing a better understanding of the role of RPE cells in retinal degenerative disorders. Identification of the complementary deoxyribonucleic acid and proteins detected by the monoclonal antibodies may provide the framework to evaluate specific RPE cell functions. RPE cell transplantation to correct retinal degenerative processes is being actively investigated in a number of laboratories. The studies reported here provide the framework to evaluate RPE cell transplantation.

Proposed Course

(1) We will continue to characterize these antibodies as well as the effect of these antibodies on cell function *in vivo* and *in vitro*.

(2) We will isolate, propagate, and characterize RPE cells for transplantation studies in animals and man. We will design effective ways to maintain the cell in culture and design ways to measure and monitor cell function.

NEI Research Program

Retinal Diseases—Photoreceptors and Pigment Epithelium

Publications

Kutty RK, Nagineni CN, Kutty G, Hooks JJ, Chader GJ, Wiggert B: Increased expression of heme oxygenase-1 in human retinal pigment epithelial cells by transforming growth factor-beta. *J Cell Physiol* 159:371-378, 1994.

Kutty RK, Kutty G, Nagineni CN, Hooks JJ, Chader GJ, Wiggert B: RT-PCR assay for heme oxygenase-1 and heme oxygenase-2: A sensitive method to estimate cellular oxidative damage. *Ann NY Acad of Sci*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00240-08 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Virus Infections in the Eye

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI
Others:	Caroline Percopo	M.S.	Biologist	LI, NEI
	Yun Wang	M.D.	Guest Worker	LI, NEI
	Miguel Burnier	M.D.	Visiting Scientist	LI, NEI
	Ingeborg Kirch	M.D.	Guest Worker	LI, NEI
	Yusuke Komuraski	M.D.	Guest Worker	LI, NEI

COOPERATING UNITS *(if any)*

Department of Pathology, The George Washington University Medical Center (Barbara Detrick, Ph.D.); Department of Pathology, Uniformed Services University of the Health Sciences (Katherine Holmes, Ph.D.); Department of Ophthalmology, Ruprecht-Karl's University, Heidelberg, Germany (Ellen Kraus-Mackiw, M.D.); Laboratory of Biology, NCI, NIH (Charles H. Evans, M.D., Ph.D.); Department of Medicine, The Johns Hopkins Medical School, Baltimore, MD (William Burns, M.D.); Laboratory of Clinical Investigations, NIAID, NIH, (Jeffrey I. Cohen, M.D., and Steven Strauss, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunology and Virology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our studies of various virologic and immunopathologic processes that occur when viruses replicate in the ocular microenvironment comprise four areas: (1) coronavirus infection in ocular and optic nerve cells; (2) the possible roles of viruses in human diseases; (3) molecular diagnosis and pathogenesis of cytomegalovirus (CMV) infections in humans, and (4) Varicella - zoster virus infections of the retina.

We have established that murine coronavirus can induce ocular disease and may be used as a model system for studying retinal degenerative diseases. This model has many unique features. The virus is capable of inducing an acute infection in the presence of mild retinal vascular inflammation. Initial retinal damage is followed by clearance of infectious virus and progressive retinal degeneration. *In situ* hybridization techniques identified that the viral RNA persists within the Müller cells and RPE cells throughout the course of the disease. Recent studies show that there are genetic and immunologic components to this disease. The retinal degenerative pathologic manifestations of the disease can be influenced by the genetics of the host. That is, some strains of mice are resistant to virus-induced retinal degenerative changes. The pathologic changes are also closely related to the development of anti-retinal and anti-RPE antibodies. These findings suggest a role for autoimmunity in the pathogenesis. This disease may be considered a model for degenerative diseases of the pigment epithelium and photoreceptors in humans.

Human CMV is a herpesvirus that is a major cause of blindness in children born with congenital infections and in immunocompromised individuals. It is difficult to study CMV latency in man. Therefore cell culture models of CMV replication and latency may provide insight into a rationale for alternative treatment modalities. We identified that CMV replicates in human RPE cells. Virus replication is extremely slow and is associated with a low expression of IE viral proteins. These may be critical variables in viral persistence and viral activation within the retina.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

This project was designed to determine the various effects of virus infections on the ocular microenvironment and to study modes of antiviral therapy.

Methods

This study involves the propagation and quantitation of viruses such as herpes simplex virus type 1, coronaviruses, and cytomegalovirus (CMV) *in vitro* and *in vivo* as well as immunocytochemical analysis of infected cells and tissues. Techniques used in the characterization of virus infection include flow cytometric analysis, Western blot analysis, Northern blot analysis, *in situ* hybridization, and amplification of viral genes by polymerase chain reaction (PCR). Techniques used in characterization of antivirus antibodies include enzyme-linked immunosorbent assay and neutralization assays.

Major Findings

(1) Coronavirus Infection in the Eye. The murine coronavirus, mouse hepatitis virus (MHV), JHM strain, induces a retinal degenerative disease in adult Balb/c mice. In the early, acute phase one to seven days after inoculation, a mild retinal vasculitis is observed. The second stage is seen by day 10 and progresses for several months. This stage is characterized by a retinal degeneration in the absence of vasculitis or inflammation. This degenerative process is associated with a reduction of the photoreceptor layer, loss of interphotoreceptor-binding protein, abnormalities in the retinal pigment epithelium (RPE) and retinal detachments. The development of the degenerative phase of the disease is controlled by a genetic predisposition of the host and is associated with the development of antiretinal and anti-RPE cell autoantibodies. During the past year, we have evaluated three aspects of this disease process: (a) virus persistence within the retina, (b) immunologic aspects of the disease, and (c) genetic predisposition to the disease.

One of the intriguing aspects of this disease process is the nature of viral clearance. The acute phase of the disease is associated with the presence of viral proteins and the detection of infectious virus within the retina. However, after day eight, infectious virus and viral proteins are not detected. Nevertheless, the retinal tissue damage characterized as retinal degeneration becomes apparent at day 10 and continues for months. The purpose of this study was to determine if the virus persists within the retina and other tissues during the course of this disease process. *In situ* hybridization was selected as a way to determine if the virus persists and to identify the cellular location of this persistent infection.

The presence of viral ribonucleic acid (RNA) was detected by *in situ* hybridization with a viral complementary deoxyribonucleic acid (cDNA) probe, and viral proteins were identified by immunocytochemical staining. cDNA probe representing MHV-A59 genes 4-6 was prepared by digesting the plasmid DNA (clone 2-2) with Pst 1. cDNA was labeled with digoxigenin-11-dUTP and hybridized with tissue sections. The resultant hybridized probe was identified with antidigoxigenin antibody conjugated to alkaline phosphatase. When uninfected (normal) mouse eye sections were incubated with the viral cDNA probe, no reactivity was observed.

In contrast, when JHM virus-infected mouse eye sections were incubated with the viral cDNA probe; positive reactivity was noted within the retina from day one to day 60 postinoculation. During the acute phase of the infection, viral RNA was found in the retina, RPE, ciliary body epithelium, and the iris epithelium. During the late phase of the infection, viral RNA was almost exclusively found within the retina and RPE and not in the anterior segment of the eye. Within the retina, viral RNA was detected in the ganglion cell layer, the inner retina, the outer retina, and the RPE cell. Pretreatment of infected eyes with RNase inhibited the reactivity and

incubation of infected eyes with plasmid cDNA resulted in no reactivity. Immunocytochemical staining identified viral protein within the retina only from day one to day eight. This ocular disease is also associated with a persistent systemic infection. Both viral RNA and viral proteins were identified within the liver during the first eight days. However, only viral RNA is detected in the liver from day eight to 60. These studies show that MHV establishes an acute infection (days one to eight) where infectious virus and viral proteins are identified. This is followed by a persistent infection within the retina and liver where only viral RNA can be detected by *in situ* hybridization.

The coronavirus-induced retinopathy model allows us to explore some of the cellular and molecular mechanisms involved in the autoimmune aspects of retinal tissue damage. During the past year, we have characterized the autoantibodies associated with the retinal degenerative process. BALB/c mice were inoculated by the intravitreal route with $10^{4.5}$ $TCID_{50}$ / 5 μl of virus or media. At varying times after inoculation, sera and eyes were removed. The presence of antiretinal and anti-RPE cell antibodies were identified by immunocytochemical staining on normal rat eye sections and by immunoblot analysis. Sera collected from BALB/c mice from 12 to 70 days after JHM virus infection contained antiretinal autoantibodies.

These antibodies are not found in the sera from normal or mock-injected mice. Antibodies to retinal tissue were identified as two distinct patterns of autoantibodies—retinal and RPE autoantibodies. Absorption studies were performed to characterize the autoantibodies. Absorption of sera with 10 percent retina, brain, or kidney tissue did not alter antiretinal or anti-RPE cell autoantibody reactivity. However, absorption of sera with lyophilized soluble fractions of rat or cow retina did inhibit both antiretinal and anti-RPE autoantibodies. Incubation of sera with lyophilized soluble fractions of rat kidney or liver did not inhibit autoantibody reactivity. The studies identify that the autoantibodies induced by the virus infection react with soluble proteins identified in the rat and cow retinas. This animal model of postvirus retinopathy is associated with the production of retinal-specific autoantibodies and may provide insight into the study of humoral autoimmune responses in human retinal degenerations.

Because the genetic composition of the host and the virus can determine the response to infection and the resulting pathology, the third phase of our studies evaluated the effect of MHV infections on different strains of mice. We reported in the past year that the pathologic manifestations of a virus infection in the retina can be influenced by the genetics of the host. BALB/c mice developed both the retinal vasculitis and the retinal degenerative phases of the disease, whereas CD-1 mice developed only the early retinal vasculitis and were spared the degenerative disease. During the past year, we have found that the A59 virus strain as well as the JHM virus strain both induce a biphasic retinal disease.

In contrast, the inoculation of MHV-3 strain by the intravitreal route did not result in a retinal degenerative disease. Within three to five days, all of the animals died. Evaluation of the brain did not reveal pathologic damage. However, the liver contained pathologic changes consistent with fulminant acute hepatitis. These remarkably different diseases are induced by different strains of the same virus. The ability of different MHV strains to cause fatal acute hepatitis or persistent retinal disease may help to decipher the mechanisms of viral tissue tropism in this strain-specific pathogenesis. These studies demonstrate that the genetics of the virus can profoundly affect the pathology generated by a virus using the eye as a portal of entry.

In summary, this model is characterized by the replication of JHM virus in the retina; producing an acute necrotizing disease of the sensory retina, resulting in only a mild inflammatory response and a long-lasting disease (longer than 14 weeks). These studies identify that a progressive degenerative disease in the retina may be initiated by an acute virus

infection in the absence of major inflammatory response. These studies during the past year clearly indicate that this retinal degenerative process has a persistent viral component, an immune component, and a genetic component. How these genetic and immunologic factors interact to influence the development of retinal degenerations are the intriguing aspects of this model.

(2) Possible Role of Viruses in Human Eye Diseases. We have initiated studies to evaluate the possible involvement of viruses in the pathogenic processes of a variety of human eye diseases. We are now collecting serum samples and ocular tissue to use seroepidemiologic approaches to detect virus and viral antigens via immunocytochemical staining, *in situ* hybridization, and PCR assays.

(3) Cytomegalovirus replication within the retina. CMV infections are frequent complications in kidney and bone marrow transplant patients and human immunodeficiency virus patients. The mechanisms by which CMV is activated and replicates within the retina is not known. We evaluated the ability of human CMV to initiate replication in human RPE cells and compared this with studies in human fibroblasts (HEL) and human amnion epithelial (WISH) cells. Human RPE cells were obtained from donor eyes and propagated *in vitro.* Cells were infected with CMV (AD169 strain) at an input multiplicity of 1. CMV replication was evaluated by immunofluorescence, flow cytometry, Western blot analysis, and infectivity assays. Cellular protein expression was evaluated by immunofluorescence and flow cytometry with monoclonal antibodies. CMV induces a cytopathic effect, infectious virus in RPE cells, and human fibroblast cells but fails to replicate in another epithelial cell—WISH cells. CMV replication in RPE cells is characterized by a prolonged period of low-virus protein expression. Less than one percent of the cells contain viral proteins (IE, E, L) during the first 10 days. These viral proteins are not detected by flow cytometry or Western blot analysis until 15 days after inoculation. In contrast, CMV proteins are found in HEL cells within 24 hours. Untreated human RPE cells *in vitro,* express MHC class I molecules, β-2 microglobulin, and intracellular adhesion molecule 1 (CD-54). The CMV infection of RPE cells results in a downregulation in the expression of MHC class I molecules on the RPE cells. Cytotoxic T cells recognize the virus-infected cell only in association with MHC class I molecules on the cell surface. This study demonstrates that CMV can replicate slowly within the RPE cell and that this replication can be monitored by flow cytometry. CMV replication in RPE cells is associated with the modulation of cellular protein expression, and this alteration may contribute to viral persistence within the retina.

(4) Varicella-zoster virus (VZV) infections of the retina. VZV is a herpes virus that is frequently associated with acute retinal necrosis and other forms of retinal tissue damage. Nevertheless, there are no good *in vivo* or *in vitro* models to evaluate virus replication and latency. During the past year, we have initiated studies to develop models of VZV infection of the retina and retinal cells. Preliminary studies indicate that human VZV can replicate in the guinea pig retina, and this replication is associated with a chronic uveitis. *In vitro* studies indicate that VZV can replicate within human RPE cells. These preliminary findings suggest that we now have two approaches to evaluate VZV replication in retinal tissues.

Significance to Biomedical Research and the Program of the Institute

Elucidating the factors involved in viral spread and pathogenesis will yield a better understanding of diseases of viral etiology. We have established a new virus model for retinal degenerative processes in adult animals. This model has many unique features. The virus is capable of inducing an acute infection in the presence of mild retinal vascular inflammation. The initial retinal damage is followed by persistence of viral RNA and progressive retinal destruction, even months after infectious virus is gone. Moreover, the development of retinal degenerative process is determined by the genetics of the host and

involves the development of antiretinal autoantibodies. This model should assist us in understanding the pathogenesis of selected human diseases of unknown etiology.

We have developed new systems to evaluate two herpes virus infections of the retina, CMV, and VZV. We have shown that CMV replicates within RPE cells in a slow, limited manner. The evaluation of the molecular aspects of this defect may provide critical clues in terms of the virus' ability to establish persistent infections and the factors initiating viral activation within the retina. We have developed two new approaches to evaluate VZV replication within the retina.

Proposed Course

(1) We will continue to evaluate coronavirus infections of the eye. The role of genetic factors and autoantibodies in the pathogenesis of retinal degenerations will be evaluated. The data obtained will be correlated with what is known about human retinal degenerative disorders.

(2) We will initiate studies to determine whether certain viruses can replicate in retinal tissues and cells. Infected cells will be evaluated for the release or expression of uveitogenic proteins.

(3) We will continue to collect samples and initiate studies to detect the involvement of viruses in human eye diseases.

(4) We will evaluate the molecular diagnosis and pathogenesis of CMV and VZV infections in the eye.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Burnier M, Wang Y, Detrick B, Hooks JJ: Retinal manifestations of a murine coronavirus infection: A histopathological and ultrastructural study. *Exp Pathol*, in press.

Hooks JJ: Ocular Virology, in Tabarra K (ed): *Infections of the Eye.* Boston, Little, Brown & Co. Publishers, 1993.

Hooks JJ, Wang Y, Komurasaki Y, Percopo C, Nagineni CN, Detrick B: Molecular and immunologic mechanisms involved in coronavirus induced retinopathy, in *Proceedings of the Sixth International Symposium on the Immunology and Immunopathology.* Amsterdam, Excerpta Medica, Elsevier Science Publishers, 1994.

Wang Y, Percopo CM, Burnier MN, Detrick B, Hooks JJ: Genetics of the virus determines retinal tissue damage induced by coronaviruses, in *Proceedings of the Sixth International Symposium on the Immunology and Immunopathology.* Amsterdam, Excerpta Medica, Elsevier Science Publishers, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00277-03 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Retinal Pigment Epithelium in Retinal Disorders

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Chandrasekharam N. Nagineni	Ph.D.	Visiting Scientist	LI, NEI
Others:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI

COOPERATING UNITS *(if any)*

Department of Pathology, George Washington University, Washington, DC (Barbara Detrick, Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunology and Virology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The retinal pigment epithelium (RPE) plays a critical role in the regulation of retinal and choroidal function in normal and disease states. Due to limited availability of human tissues, an *in vitro* cell culture system is desired. Therefore, we have developed and characterized the primary cell lines of human RPE from donor eyes obtained from eye banks. Using human RPE cell cultures as a model, we conducted investigations to examine the various roles of RPE in the pathophysiology of retinal disorders.

Human RPE cultures secreted significant quantities of interleukin-6 (IL-6) and intercellular adhesion molecule-1 (ICAM-1) but not interleukin-1 (IL-1) in response to the stimulation by inflammatory mediators. Interferon gamma (IFN-γ) exhibited synergistic effects in the secretion of IL-6 and ICAM-1 in the presence of suboptimal levels of tumor necrosis factor α (TNF-α) or IL-1. Cellular expression of ICAM-1, mostly localized to intercellular junctions, was observed in RPE treated with TNF-α and IFN-γ. There is a close correlation between IL-6 and ICAM-1 secretion and IL-6 and ICAM-1 mRNA levels, respectively, suggesting the regulation at the gene transcription. The responses of RPE to inflammatory mediators in IL-6 and ICAM-1 secretion was rapid and sustained in the presence of stimulants but reversed to control levels quickly upon withdrawal of the stimulants, indicating the reversibility of the responses of RPE to inflammatory signals. These results show that RPE responds to inflammatory stimuli by increased cellular expression and secretion of IL-6 and ICAM-1, which may in turn perpetuate immune reactions in the pathogenesis and/or prevention of retinal and choroidal diseases.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Krishnan R. Kutty	Ph.D.	LRCMB, NEI
Barbara Wiggert	Ph.D.	LRCMB, NEI

Objectives

The primary objectives of this research project are to: (1) establish primary cell lines of human retinal pigment epithelial (HRPE) cells from donor eyes, (2) develop serum-free media and other factors that can induce differentiation and pigmentation of HRPE, (3) investigate the role of inflammatory mediators and growth factors on cellular and molecular aspects of HRPE structure and function, and (4) evaluate the usefulness of HRPE and rat retinal pigment epithelial (RPE) cultures for transplantation studies to restore retinal functions in hereditary, autoimmune, and age-related disorders.

Methods

Primary cell cultures of HRPE are prepared by initial seeding of either freshly isolated RPE cells or RPE-choroid explants. Cells are grown in minimum essential medium supplemented with 10 percent fetal calf serum and nonessential amino acids and antibiotics. We are developing serum-free, hormonally defined media and extracellular matrix factors that would induce differentiated morphology for the RPE cells.

Techniques required for cell culture, immunofluorescence, cytokine, and intercellular adhesion molecular 1 (ICAM-1) assays by enzyme-linked immunosorbent assay, gel electrophoresis, Western and Northern blotting for proteins and ribonucleic acid (RNA), reverse transcription polymerase chain reaction (RT/PCR) are developed and standardized in our laboratory to carry out these studies.

Major Findings

In the past, age of the donor was considered very critical in preparing human RPE cultures because eyes from donors older than 50 years of age did not yield fruitful cell lines, probably due to senescence-associated loss of viability. In these experiments, RPE cells were first disassociated from the eye cups by digestion with proteolytic enzymes, a treatment that might have caused initial contamination with nonepithelial cells from which it is impossible to purify epithelial cells. Therefore, we have developed a new method using RPE-choroid explants to initiate cell growth. By careful monitoring of clusters of cells growing around the explants, we were able (on the basis of morphology combined with experience) to select pure epithelial cells and discard nonepithelial cells at the primary culture stage.

Using this technique, we established primary cell lines of human RPE from eyes obtained from 81- and 87-year-old donors. The epithelial nature of these cell lines was confirmed by immunochemical staining for cytokeratin with monoclonal antibodies. All of the cells expressed cytokeratin at different passages. Immunoblotting analysis of cellular proteins indicated cytokeratin-18 was the predominant cytokeratin in these cells. Because RPE is the only epithelial cell in the posterior segment (choroid-RPE-retina), these results establish without doubt that the cell lines developed are, in fact, RPE.

Human RPE cultures secrete significant quantities of interleukin (IL)-6 and ICAM-1, but not IL-1, in response to the stimulation by inflammatory mediators. IL-1 is the most potent stimulant of IL-6 secretion followed by tumor necrosis factor alpha (TNF-α) and lipopolysaccaride (LPS). Interferon gamma (IFN-γ) had only minimal effects on IL-6 secretion. In contrast to IL-6 secretion, ICAM-1 secretion was not influenced by LPS. TNF-α, IFNγ, and IL-1 had almost similar effects on ICAM-1 secretion by HRPE. The effect of IFN-γ is striking in that it can act synergistically in the presence of suboptimal levels of

TNF-α or IL-1 to augment secretion of both IL-6 and ICAM-1. More than 98 percent of IL-6 produced by HRPE cells was secreted promptly into the medium. Western blot analysis of secreted IL-6 revealed multiple molecular forms suggesting that HRPE are capable of carrying out posttranslational glycosylation processes that may be important for functional activities. Cellular expression of IL-6 was not detected by immunofluorescence staining of the cells. In contrast, intense staining for ICAM-1—that was mostly localized to intercellular junctions of the monolayer of epithelial cells—was observed in HRPE treated with TNF-α and/or IFN-γ. Analysis of IL-6 and ICAM-1 messenger ribonucleic acid (mRNA) expression by Northern blotting indicated rapid and sustained responses of HRPE to inflammatory cytokines that can be reversed quickly on withdrawal of the stimulus. There is a close correlation between IL-6 and ICAM-1 secretion as well as IL-6 and ICAM-1 mRNA levels, respectively.

Our results indicate that HRPE respond to LPS and inflammatory cytokines (TNF-α, IL-1, and IFN-γ); and enhance IL-6 and ICAM-1 gene expression and secretion of proteins. During posterior uveitis of the eye caused by infections or autoimmune diseases, macrophages and lymphocytes infiltrate into the retina and secrete cytokines such as TNF-α, IL-1, IFN-γ, and IL-2 that would initiate immune reactions. These cytokines in their turn act on retinal resident cells to locally produce IL-6 and ICAM-1 to amplify the immunopathological processes. IL-6, a multipotent cytokine, plays a major role in the autoimmune and inflammatory disorders by its ability to induce proliferation and differentiation of lymphocytes and production of antibodies. ICAM-1, an adhesive glycoprotein, participates in inflammatory reactions by recruiting leukocytes to the sites of inflammation, lymphocyte proliferation, cytotoxic T-cell function, and T-cell mediated B-cell activation.

Several lines of evidence support that bacterial endotoxins and cytokines TNF-α, IL-1, IFN-γ, IL-2, and IL-6 play a critical role in uveitis and other inflammatory diseases of the eye. Intravitreal injection of IL-1, TNF-α, or IL-6 has been shown to cause uveitis in experimental animal models. Moreover, elevated levels of IL-6, IFN-γ, IL-1, and TNF-α have been found in aqueous humor and vitreous aspirates of patients with uveitis, proliferative vitreoretinopathy, and other noncomplicated retinal detachments. Upregulation of the expression of ICAM-1 on retinal cells and epiretinal membranes and an increase in soluble ICAM-1 in vitreous of patients with inflammatory retinal diseases suggest a vital role for ICAM-1 in various diseases. Our studies suggest that RPE reacts to inflammatory stimuli and secrete IL-6 and ICAM-1, thereby elevating these proteins in the local environment for the perpetuation of immunopathological processes. The synergistic actions of IFN-γ on IL-6 and ICAM-1 secretion by HRPE cells in the presence of other cytokines would result in an effective amplification mechanism because several cytokines are produced simultaneously during inflammation.

The roles of growth factors, basic fibroblast growth factor, transforming growth factor beta (TGF-β) and platelet-derived growth factors in RPE functions, and the regulation of secretion of these growth factors by RPE are being investigated. We found that these growth factors had no effect on the expression and secretion of IL-6, ICAM-1, and IL-1. The expression of heme oxygenase-1 (HO-1) was increased by TGF-β in HRPE cells by fourfold to fivefold within hours of stimulation. However, LPS, inflammatory cytokines, and other growth factors had no effect on HO-1 levels. Among ocular tissues, RPE has the highest activity of HO-1. HO-1 catalyzes the oxidation of heme into biliverdin and carbon monoxide. Biliverdin is converted by nonlimiting enzymatic reaction into bilirubin, an antioxidant that offers protection of cells against heat and oxidative stress. These results suggest that TGF-β upregulates defense mechanisms of RPE, a phagocytic cell that is constantly subjected to chemical stress, by engulfing the shed outer segments of retinal rods and cones for protection against oxidative damage.

Significance to Biomedical Research and the Program of the Institute

Primary cell lines of human RPE are an ideal *in vitro* model for evaluation of several functions of RPE and for further elucidation of the mechanisms of RPE involvement in the pathogenesis of retinal and choroidal diseases. These cells are potentially useful in cellular transplant therapy to correct hereditary and age-related macular degeneration defects in humans.

Proposed Course

Two of the major problems associated with human RPE cell cultures are: (1) progressive loss of pigmentation on serial passaging of cells and (2) lack of clear intercellular junctions and *in vivo*-like morphological appearance. These changes could probably be due to cytoskeletal reorganization and partial dedifferentiation. Development of such fully differentiated RPE cell lines is crucial not only to understanding cellular functions but also for cellular transplant therapy. Our goal is to examine the mechanisms by which RPE cultures can be induced to resume *in vivo* characteristics. Preliminary studies show that HRPE cells assume differentiated morphology on incubation in serum-free medium containing insulin, transferrin, selenium, hydrocortisone, prostaglandins, and tri-iodothyronine. Further studies will be conducted in defining and selecting specific media composition and/or culturing on suitable extracellular matrix.

The effects of inflammatory cytokines and bacterial endotoxins on HRPE will be evaluated: (1) on the secretion and expression of IL-8 and granulocyte-macrophage colony-stimulating factors; (2) on the role of anti-inflammatory cytokines TGF-β, IL-4, and IL-10 on the influences of inflammatory mediators; (3) on cellular cytoskeletal organization, intercellular junctions, and adhesion properties; and (4) characterization of growth factors and proteolytic enzymes secreted by RPE in response to various stimuli. These studies are likely to shed light on the role of RPE in the pathophysiology of retina and choroid, the tissues that are in close vicinity to and have direct influence on RPE.

NEI Research Program

Retinal Diseases—Inflammatory Diseases, Macular Degeneration, Photoreceptors and Pigment Epithelium

Publications

Kutty RK, Nagineni CN, Kutty G, Hooks JJ, Chader GJ, Wiggert B: Increased expression of heme oxygenase-1 in human retinal pigment epithelial cells by transforming growth factor-b. *J Cell Physiol* 159:371-378, 1994.

Nagineni CN, Detrick B, Hooks JJ: Synergistic effects of gamma interferon on inflammatory mediators that induce interleukin-6 gene expression and secretion by human retinal pigment epithelial cells. *Clin Diagnos Lab Immunol*, (in press).

Nagineni CN, Detrick B, Rhame J, Hooks JJ: Inflammatory cytokines IFN-γ, TNF-α and IL-1 induce ICAM-1 secretion/shedding by human retinal pigment epithelial cells. Invest. Ophthalmol. *Vis Sci* 35 (4):2040, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00287-02 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Toxoplasmosis Infections in the Eye

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI
Others:	M. Cristina Martins	M.D.	Guest Worker	LI, NEI
	Chandrasekharam Nagineni	Ph.D.	Visiting Scientist	LI, NEI
	Miguel Burnier	M.D.	Visiting Scientist	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	LI, NEI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunology and Virology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Toxoplasma gondii infections are a major source of visual loss and blindness. Ocular toxoplasmosis may occur as a result of congenital infections, acquired infections, and as a manifestation of immunosuppression, particularly as a result of transplantation or acquired immunodeficiency syndrome (AIDS). Due to the recent resurgence of acquired ocular toxoplasmosis in Brazil and the worldwide complications of toxoplasmosis in HIV infections, we initiated studies to develop a model of acquired toxoplasmosis to evaluate the molecular mechanisms of pathogenesis and therapeutic strategies.

We have developed a murine model of ocular toxoplasmosis that is characterized by retinal inflammation, chorioretinal scaring, retinal disorganization, and cyst formation. Retinal disease occurs in three different strains of mice following inoculation with toxoplasmosis by the subcutaneous or intraperitoneal routes. This model of acquired ocular toxoplasmosis is being used to evaluate the efficacy of new antiparasitic agents in controlling the development of retinal cyst formation and retinal inflammation.

Project Description

Objectives

This project was designed to develop an animal model of acquired ocular toxoplasmosis and *in vitro* models of toxoplasmosis replication within the retina. These models will be used to evaluate molecular mechanisms of ocular pathogenesis and to evaluate new antiparasitic drugs and cytokines.

Methods

This study involves the propagation and quantitation of *Toxoplasmosis gondii* (*T. gondii*) strains *in vitro* and *in vivo*, as well as immunocytochemical analysis of infected cells and tissues. Techniques used in the characterization of *T. gondii* infections include histopathology, immunocytochemistry, *in situ* hybridization, and Western blot analysis. Techniques used in characterization of anti-*T. gondii* antibodies include enzyme-linked immunosorbent assays.

Major Findings

Adult Swiss Webster, C57BL6 and BALB/C mice were inoculated by the subcutaneous route or intraperitoneal route with 10 *T. gondii* cysts (S2C9 or ME49 strains) in a 1 mL volume. At various times after inoculation (day seven, 14, 21, 28, and 42) the mice were sacrificed, and eyes and brains were removed and fixed in 10 percent buffered formalin. Fifteen hematoxylin and eosin sections of brain and eye were evaluated for the presence of *T. gondii* cysts.

By day 14, 100 percent of the mice developed cysts in the brain. Retinal inflammation was also noted in 100 percent of the animals by day 14. Chorioretinal scars were also observed in mice inoculated with both strains of *T. gondii*. Retinal cysts were found in mice 28 and 42 days after inoculation with ME49 strain and 14 and 42 days after inoculation with S2C9 strain in Swiss Webster mice. *T. gondii* cysts in the retina were also detected in C57BL6 mice at 14, 21, 28, and 42 days after inoculation with the S2C9 strain. This study identifies an animal model of ocular toxoplasmosis characterized by retinal inflammation, chorioretinal scaring, retinal disorganization, and cyst formation.

Preliminary studies on an *in vitro* cell culture model for *T. gondii* replication in retinal tissues has revealed that *T. gondii* can infect and replicate in human retinal pigment epithelial cells. Initial studies also indicate that this replication can be inhibited by the addition of recombinant human interferon gamma.

Significance to Biomedical Research and the Program of the Institute

This is the first animal model of acquired toxoplasmosis that consists of retinal inflammation, degeneration, and parasitic cyst formation. This model will allow us to evaluate the efficacy of new antiparasitic drugs in controlling the development of retinal cyst formation and retinal inflammation and scarring.

Proposed Course

We will evaluate drugs and cytokines in controlling the ocular manifestations of acquired *T. gondii* infections.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00293-01 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene Targeting of Invariant Chain Gene: A Tool To Study Immunoregulation in Autoimmune Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Moncef Jendoubi	Ph.D.	Visiting Scientist	LI, NEI
Others:	Noriko Esumi	M.D., Ph.D.	Visiting Associate	LI, NEI
	Daniel H. Lacorazza	Ph.D.	Visiting Fellow	LI, NEI
	Luis J. Rivero	Ph.D.	Visiting Fellow	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section of Genetics and Molecular Immunology

INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.9	3.9	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Class II antigens of the major histocompatibility complex (MHC) are essential in the immune response because they bind processed polypeptides for presentation to T lymphocytes. The interactions of class II MHC molecules on the antigen-presenting cell with a responding T lymphocyte are complex because they involve molecular contacts with an antigen peptide, an antigen-specific T-cell receptor, and the CD4 molecule expressed on the T lymphocytes. Most antigens must be processed in order to bind to MHC molecules and to be recognized by T lymphocytes.

In this context, the majority of peptides bound by MHC class II molecules during antigen loading are not derived from ingested and processed foreign proteins but instead are primarily derived from a finite number of self-proteins. During progression outward through the exocytic pathway in the process of antigen presentation, class II-invariant chain complexes play a critical role.

Experimental autoimmune uveoretinitis (EAU) in rodents is a T-cell-mediated autoimmune response, particularly against the photoreceptors of the neural retinal cells, and can serve as a model for human uveitis. The roles of MHC and non-MHC genes have been strongly associated with EAU in rats and mice. To further study the MHC class II participation in mice animal model, we decided to generate deficient mice for the invariant chain gene (li). This glycoprotein combines with MHC class II heterodimers from the beginning of their synthesis in the endoplasmic reticulum, travels through the Golgi apparatus, and ends up in endosomal compartments where it is either proteolytically cleaved or degraded. The absence of li has been shown to affect the transport of class II molecules, resulting in a poor antigen presentation.

Project Description

Objectives

Targeting Vector. A replacement vector has been designed to introduce a mutation in Ii murine gene, where the neomycin phosphotransferase gene was inserted in the middle of the second exon. Two fragments of 0.3 Bgl II-Sac I and 9.2 Kb Sac I-EcoR I were added upstream and downstream of the neomycin gene, respectively. Furthermore, two copies of herpes simplex virus thymidine kinase (HSV-tk) that allow the negative selection were cloned at the end of the targeting vector. The targeting fragment used for the electroporation was excised from the backbone vector by digestion with Not I, dialyzed against TE (Tris/HCl 10 mM, pH 7.5, EDTA 1 mM), precipitated with ethanol, resuspended in an appropriate buffer at a concentration of 1 mg/ml, and analysed on agarose gel.

Methods

Electroporation. Embryonic stem cells (D3, from 129/5v strain) were cultured in standard condition on an inactivated layer of embryonal fibroblasts and fed two and one-half hours before electroporation. Cells were trypsinized, washed with DMEM, and resuspended in HBS (Hepes 25 mM, NaCl 134 mM, 5 mM KCl, Na_2PO_4 0.7 mM, pH 7.1) at $2x10^7$ cells/ml. The same volume of HBS containing 100 μg/ml of targeting vector was added to the cell suspension and incubated 10 minutes on ice. The electroporation was carried out using a 600 BTX electroporator with the following conditions: Volts: 250; μFaradays: 300; Resistance: R8.

Transfected cells were left at room temperature for 10 minutes and seeded at a concentration of 5×10^6 cells/10 cm Petri dish. Twenty-four hours later, the cells were selected using G418 (200 μg active substance/ml) and ganciclovir (2 μM).

Blastocyst Injection. Blastocysts, three and one-half days old, were collected by flushing the uterus of super ovulated C57BL/6 females. Mutated cells in the Ii gene were injected into blastocysts before their transfer to pseudo-pregnant B6D2 FI foster mothers.

Major Findings

A total of 8×10^7 D3 cells were electroporated with the targeting vector and selected with G418 and ganciclovir for 10 days. Double-resistant clones were picked up individually and expanded in a 24-well plate. Deoxyribonucleic acid (DNA) was isolated from each clone and analyzed by polymerase chain reaction (PCR) for homologous recombination events, using a combination of primers that allow the discrimination of random integrations. In addition, genomic DNA was isolated from all double-resistant clones and analyzed by Southern blot, using both inside and outside probes for the invariant chain gene. For further confirmation, the same studies were repeated, using various restriction enzymes. Taken together, the results of PCR and Southern blot analysis confirmed that three out of 130 double-resistant clones scored positive.

These three targeted clones were further expanded and injected into blastocysts, and the embryos were later transferred into the foster mothers. Offspring carrying the mutated Ii gene were identified by their chimeric coat color. Again, DNA was purified from all chimeric mice and analyzed by PCR as well as by Southern blot. The obtained results showed that the targeted mutation has been transmitted to the offspring. Heterozygous mice were set up for mating to generate homozygous mice mutated on both alleles of the invariant chain gene. Presently, we were able to create homozygous mice and to establish a colony of these animals. Homozygous mice born in the colony are normal and grow up without showing any obvious abnormality.

Significance to Biomedical Research and the Program of the Institute

The creation of a deficient mouse for the invariant chain gene represents a previously unavailable tool to study several important

phenomenons in the immune system, both in normal and pathological conditions.

Degenerative and inflammatory diseases of the posterior pole of the eye are common causes of impaired vision and blindness throughout the world. Between 500,000 and 1,000,000 Americans suffer severe visual impairment from retinal and choroidal diseases. Retinal degenerative disorders consist of a diverse group of diseases frequently associated with a genetic predisposition such as major histocompatibility complex (MHC) class II. However, in many ocular diseases the causes are unknown. In this respect, invariant chain-deficient mice will be very useful to study some of these ocular disorders. Thus, these mice will be used to study the implication of MHC class II genes in ocular immunopathological diseases.

Proposed Course

In the future, we will focus our work on the following:

(1) We will use the invariant chain gene-deficient mice to study endotoxin-induced uveitis.

(2) We will study the effect of viral infection such as murine coronavirus that induces an acute, long-lasting disease of the retina to clarify the degenerative and inflammatory diseases that affect the retina and the choroid in many ocular disorders (this study will be conducted in collaboration with Dr. John Hooks in the LI).

(3) We will use these deficient mice as models to study allergic conjunctivitis, in collaboration with Dr. Chi-Chao Chan in the LI.

(4) We will use these deficient mice as models to study experimental autoimmune uveitis, in collaboration with Dr. Rachel Caspi in the LI.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Lacorazza DH, Rivero LJ, Jendoubi M: Expression of the human OAT gene after retroviral transfer into CHO deficient cell line. *NIH Research Festival* E05:35, 1993.

Rivero LJ, Jendoubi M: Creation of invariant chain gene deficient mice as a tool to study autoimmune disease and uveitis. *Sixth International Symposium on Immunology and Ophthalmology*, NIH, June 22, 1994.

Rivero LJ, Jendoubi M: Gene targeting of invariant chain gene by homologous recombination as a tool to study immunoregulation in autoimmune diseases. *Advances in Ocular Immunology*, p. 163, Amsterdam, Netherlands, Elsevier Press, 1994.

Rivero LJ, Jendoubi M: Embryonic stem cell lines carrying insertional mutation. *Cold Spring Harbor M Mol Gen*, p 172, 1992.

Rivero LJ, Jendoubi M: Mutagenesis of ESC and generation of chimeric mice. *NIH Research Festival* 4:23, 1992.

Rivero LJ, Kozhich A, Nussenblatt RB, Jendoubi M: Retrovirus expression of ornithine δaminotransferase in vitro toward gene therapy. *Invest Ophthalmol* 34(4):807, 1993.

Rivero LJ, Kozhich A, Nussenblatt RB, Jendoubi M: Retrovirus-mediated gene transfer and expression of human ornithine δ-aminotransferase into embryonic fibroblast. *J Cell Biol* 17E:251, 1993.

Rivero LJ, Lacorazza DH, Kozhich A, Nussenblatt RB, Jendoubi M: Retrovirus-mediated gene transfer and expression of human ornithine delta transferase into embryonic fibroblasts: An alternative approach to somatic gene therapy. *Hum Gen Ther* 5:701, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00292-01 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Retinal Survival in Transgenic Mice Expressing Human Ornithine ∂-Aminotransferase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Moncef Jendoubi	Ph.D.	Visiting Scientist	LI, NEI
Others:	Noriko Esumi	M.D., Ph.D.	Visiting Associate	LI, NEI
	Daniel H. Lacorazza	Ph.D.	Visiting Fellow	LI, NEI
	Luis J. Rivero	Ph.D.	Visiting Fellow	LI, NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunology	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section of Genetics and Molecular Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.9	3.9	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Gyrate atrophy (GA) is a severe human recessive eye disease resulting in progressive loss of vision due to chorioretinal degeneration. The disorder is associated with a deficiency of the mitochondrial matrix enzyme, ornithine δ-aminotransferase (OAT), which catalyzes the interconversion of ornithine and α-ketoglutarate to Δ-pyrroline-5′carboxylate and glutamate. GA patients with activity have 10- to 20-fold higher levels of plasma ornithine as compared with controls. Dietery and specific hormonal administration in rats and mice have been shown to modulate the regulation of this enzyme in different tissues, suggesting OAT's important physiological role in vivo. To test this hypothesis and to find out whether ornithine δ-aminotransferase could be a common mediator for retinal cell growth, we produced two transgenic lines, expressing the human OAT gene in strains of mice normally exhibiting a progressive retinal degeneration. Here we show that transgenic mice expressing human ornithine δ-aminotransferase exhibit less severe retinal degeneration than control mice littermates.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

Certain inbred strains of mice, such as FVB/N, present a progressive retinal degeneration beginning a few days after birth and becoming complete a few months later. Retinal degeneration in these mice has been associated at least in part with a deficiency in a phosphodiesterase activity, which leads to the accumulation of cyclic GMP in affected retina degenerative photoreceptors. We postulated that other gene products such as ornithine-δ-aminotransferase (OAT), which is associated with retinal degeneration in humans may be involved. Thus, persistent expression of human OAT (hOAT) in retinal degenerated mice could have a role in the development of retinal cell layers. To study the physiological relevance of OAT *in vivo* and to determine whether its expression could rescue the retinal cell layers from degeneration, we produced two transgenic lines (OATtg) expressing the hOAT gene in strains of mice normally exhibiting a progressive retinal degeneration. Therefore, it can be seen that transgenic mice expressing hOAT exhibit less severe retinal degeneration than control mice litter mates.

Methods

Production of Transgenic Mice. hOAT complementary deoxyribonucleic acid was cloned under the transcriptional element of Moloney murine leukemia retrovirus long terminal repeat (LTR-MoMuLV), and the construct was injected into zygotes of FVB/N mice strain genetic background.

Biochemical Analysis of Transgenic Mice. Cells were prepared from different tissues from both transgenic mice and control litter mates; the cells were lysed in 500 mM NaCl, 50 mM Tris pH 7.5, one percent NP40. 30 μg of protein extracts from each sample was subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and stained with coomassie blue or transferred to nylon filters for immunoblot analysis.

Histopathology. Enucleated eyes were prefixed in 4 percent phosphate-buffered glutaraldyde for one hour after being fixed in 10 percent formaldehyde overnight, dehydrated, and embedded in methacrylate. Four μm-thick sections were cut horizontally along the pupillary-optic nerve plane of the eye and were stained with hematoxylin-eosin. Sections were evaluated, and microphotographs were taken at a magnification of 400 X.

Major Findings

Biochemical Analysis. OATtg mice were born normal. The expression of the transgene was assessed primarily by Northern blot analysis and then in several tissues using immunoblot analysis and specific antibodies raised against hOAT. When the protein extracts were separated on SDS/PAGE and stained with coomassie blue, we saw the presence of new polypeptides, and/or an enhancement of several others, at all range of molecular weights only in the OATtg tissue protein extracts.

OATtg and control litter mates were analyzed for their expression of hOAT and its consequences on cell growth, particularly in the eye. The expression of the transgene was assessed primarily by Northern blot analysis and then in several tissues using immunoblot analysis. The correct size of hOAT protein in transgenic mice, as detected with specific antibodies raised against hOAT, indicated that our transgene encoded the entire protein.

Histopathology. To further determine whether the expression of hOAT would have any consequence on the retinal cell layer development, we examined histopathologically OATtg and the control litter mates derived from both founders at different timepoints. At birth, no significant differences of the retinal structure, mainly the inner and outer neuroblast layers, between wild-type and OATtg mice were observed. At one week, wild-type retina showed, as expected, an early degenerative development of the outer segments of the photoreceptors with a slight reduction of the outer and inner nuclear layers (ONL and

INL). On the contrary, OATtg retina showed better preserved inner and outer segments of the photoreceptors (IS/OS) with larger numbers of intact nuclei both in the ONL and INL.

At two weeks, the retinal thickness between the wild-type and OATtg showed remarkable differences. The ONL of wild-type retina showed no more than one row of photoreceptor nuclei, and the IS/OS had become a debris of degenerating membranes, owing to the defect in FVB/N with congenital retinal degeneration. In contrast, at least three rows of cells with relatively fewer pyknotic nuclei remained in the ONL of OATtg retina. In the transgenic mice, the thickness of the IS/OS was almost double that of the wild-type retina. The INL and the outer plexiform layer (OPL) showed better differentiation and more thickness in OATtg than in wild type.

After two months, retina in the control litter mates showed complete degeneration and atrophy with total loss of all photoreceptor cells, including the nuclei, the IS/OS, and OPL. The ONL was in direct contact with the retinal pigment epithelium (RPE). The residual single row of degenerated photoreceptor nuclei and the remains of IS/OS and OPL were identified in some regions. During the retinal development, the most striking difference between OATtg and wild type was observed at two weeks. All three wild types showed a retinal degeneration, but the OATtg mice exhibited a retardation of the degenerative process.

Significance to Biomedical Research and the Program of the Institute

Although the physiological role of OAT and how it contributes to retinal cell growth, whether directly or indirectly, remains to be understood. The present study provides strong evidence that OAT plays a critical role in rescuing neural cell lines in the retina. In the human, many ocular diseases affect both retina and choroid and lead to blindness. This is the first time that we show a physiological role of OAT and demonstrate that it participates in some extent to the survival of retinal cells. Thus, by virtue of its role as a growth-factor, OAT represents a valuable model that may aid studies of the pathogenesis and treatment of human retinal degeneration.

Proposed Course

In the future we will focus on the following:

(1) We will breed these OATtg+ mice with OAT-deficient mice to restore the missing enzymatic activity.

(2) We will study the consequences of the overexpression of the exogenous OAT on other tissues.

(3) We will try to find out how the overexpression of OAT leads to the survival of retinal cells.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

PROJECT NUMBER
Z01 EY 00294-01 LI

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Enzymatic Correction of OAT Deficiency: Progress Toward Gene Therapy to Ocular Genetic Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Moncef Jendoubi	Ph.D.	Visiting Scientist	LI, NEI
Others:	Noriko Esumi	M.D., Ph.D.	Visiting Associate	LI, NEI
	Daniel H. Lacorazza	Ph.D.	Visiting Fellow	LI, NEI
	Luis J. Rivero	Ph.D.	Visiting Fellow	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section of Genetics and Molecular Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.9	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Gyrate atrophy (GA) of the choroid and retina is an autosomal recessive chorioretinal degeneration, caused by deficiency of the mitochondrial matrix enzyme ornithine-δ-aminotransferase (OAT). This deficiency results in the accumulation of ornithine in the body fluids and leads to hyperornithinemia. Although the clinical phenotype is largely confined to the eye, OAT deficiency is a systemic disorder.

With the final goal of applying gene therapy to this human genetic disease, we have established an *in vitro* model to test the correction of OAT enzymatic deficiency in mammalian cells, using OAT-recombinant retroviruses.

Herein, we report the construction of several Moloney murine leukemia virus (MoMLV)-based recombinant retrovirus vectors, in which the human OAT cDNA was placed under the transcriptional control of the mouse phosphoglycerate kinase (PGK) promoter or under the enhancer-promoter regulatory element derived from MoMLV long terminal repeat (LTR). The retrovirus constructs were packaged in the PG13-GALV cell line and used to transduce C9, an OAT-deficient cell line derived from Chinese hamster ovary cells (CHO-K1). We showed that the recombinant retrovirus transfers the hOAT gene into C9. Expression of the hOAT gene in the transduced C9-deficient cell line exceeded the level of endogenous human fibroblasts, OAT mRNA, and enzymatic activity.

Project Description

Objectives

Ornithine-δ-aminotransferase (OAT) (L-ornithine:2-oxo-acid aminotransferase, 2.6.1.13) is a nuclear-encoded mitochondrial matrix enzyme that catalyzes the reversible interconversion of ornithine and α-ketoglutarate to glutamate semialdehyde and glutamate. OAT monomers are synthesized as 49-kDa precursors and processed to 45-kDa forms upon entry into the mitochondrial matrix where they assemble into the active homohexameric form of the enzyme. The OAT gene in humans has been characterized and mapped to the site 10q26. Both rat and human complementary deoxyribonucleic acid (cDNA) have been cloned. This has greatly facilitated the study of OAT's physiological role. In previous studies, it has been shown that gyrate atrophy (GA) patients have a high concentration of ornithine in their body fluids, up to 20 times the normal level. This hyperornithinemia was associated with the absence of OAT enzymatic activity. More recently, sequencing of OAT cDNA from GA patients revealed the presence of mutations—mostly point mutations—that cause frameshift, nonsense, and missense mutations and lead to the inactivation of the OAT gene and ultimately to the absence of enzymatic activity typical of GA disease

The hyperornithinemia associated with GA of the choroid and retina can be lowered to normal levels with a low-protein and low-arginine diet in all cases studied so far, and with pyridoxine hydrochloride (vitamin B6) in some cases. Because an arginine-free diet cannot be easily followed for a long period of time, this treatment is temporary and palliative rather than curative. With the view of applying a genetic therapy to GA patients and correcting the OAT enzymatic deficiency by supplying a functional gene, we established an *in vitro* model system in which we attempted to correct the enzymatic deficiency in an OAT-deficient cell line as a first step toward a somatic gene therapy.

Methods

We constructed several OAT-recombinant retroviruses bearing human OAT (hOAT) cDNA under different regulatory elements, transduced them into cells—an OAT-deficient cell line—and finally studied the efficiency of OAT gene expression following retrovirus-mediated transfer. Using Southern, Northern, and Western blot analyses as well as specific OAT enzymatic assays we show that OAT recombinant retroviruses efficiently transfer the gene to recipient cells leading to high expression of an active OAT enzyme.

Major Findings

Establishment and Characterization of a Recombinant Retrovirus Producer Cell Line. Four retroviral vector constructs bearing the hOAT cDNA as well as the neomycin phosphotransferase (*neo*^{R}) gene as selectable marker were packaged into virions using the PG13-GALV cell line. The PG13-GALV packaging cell line was used to produce virions with Gibbon leukemia virus host range and to infect hamster cell lines, including C9.

The retrovirus producer cells were screened for the presence of an unrearranged OAT recombinant retrovirus, the production of high-titer virus and the functional expression of the inserted hOAT cDNA. The pattern of the Southern blot indicates that the retrovirus constructs were integrated into the packaging cell genome without rearrangement. Moreover, the hOAT expression was confirmed by Northern blot analysis, and the results showed the presence of the corresponding OAT transcripts in the four transfected packaging cell lines.

To determine whether the inserted hOAT recombinant retrovirus is able to produce an active enzyme, we performed an enzymatic assay on cell lysates from PG13-GALV retrovirus producer cell lines previously transfected with the constructs described above. The four different lysates showed a high expression of OAT enzyme, however, the comparison of the enzymatic activity between them demonstrate

that the PG13-GALV retrovirus producer cell line transfected by LPOSN expresses the highest OAT activity.

Correction of OAT Deficiency in C9-Transduced Cell Line. To further assess the expression of integrated hOAT sequences, we analyzed the production of the OAT messenger ribonucleic acid (mRNA). We purified total cellular ribonucleic acid (RNA) from wild-type C9 cells and transduced ones with LPOSN retrovirus as well as from normal human fibroblasts. Total RNA from these cells was processed for Northern blot analysis. The results showed strong hybridization on a 5.0-kbp and a weaker hybridization on a 3.6-kbp transcript, which might suggest that the major transcript could be derived from the viral long terminal repeat and the minor transcript from the phosphoglycerate kinase (PGK) promoter.

This result may suggest an interference between viral and PGK promoter. The native hOAT transcript from human fibroblasts showed the corresponding size or 2.1-kbp. The hOAT transcripts in the transduced C9 cell line are in greater abundance than the endogenous hOAT mRNA in the normal human fibroblasts taking into account that the lanes contain the same amount of total RNA as confirmed by hybridization with a β-actin probe. To test if the mRNA transcript from the proviral insert was being appropriately translated and processed into a mature protein, we performed a Western blot analysis using protein extracts from transduced and nontransduced C9 as well as from human fibroblasts, and an anti-hOAT antibody directed against a 19-mer peptide located in the N-terminal domain of the OAT protein. No significant immunological reaction was observed in C9 wild-type extract, while in transduced cells a strong reaction was seen on one polypeptide, which comigrates with an apparent molecular weight of 45-kDa with that detected in human fibroblasts. From these results, we infer that the OAT produced by the transduced cells has been well processed into a mature protein.

To further assess whether the expressed OAT protein in the transduced cells is enzymatically active, cell lysates were prepared from transduced and nontransduced C9 and from human fibroblasts and were analyzed for the presence of an active OAT. The deficient cell line has almost no OAT enzymatic activity, but the human native fibroblast cells showed a normal activity (specific activity [SA] = 24 ± 2 nmol Δ^1-pyrroline-5-carboxylate [Δ^1P5C] formed/mg of protein/h), whereas the transduced C9 cells produced at least a threefold increase of OAT activity as compared with human fibroblasts (SA = 65 ± 9 nmol Δ^1P5C formed/mg of protein/h). These results show that the LPOSN provirus is capable of expressing high levels of functional hOAT enzyme that represents at least 10-fold more than the OAT residual activity in deficient cells.

Our results demonstrate that the retrovirus-mediated transfer of hOAT results in a stable integration of the transferred gene without rearrangement into the transduced cells genome. In addition, we have shown that the gene was transcribed, translated, and processed into a protein recognized by a specific hOAT antibody and able to metabolize the ornithine in its natural substrate.

Significance to Biomedical Research and the Program of the Institute

OAT deficiency is associated with hyperornithinemia and degeneration of the choroid and retina, suggesting that the accumulation of ornithine may produce a toxic effect on eye tissue.

The introduction of a normal OAT gene via retrovirus transfer into somatic cells of GA patients consequently may turn the hyperornithinemia to normal level and lead to an improvement in visual function. Retrovirus gene delivery has been extensively used in *in vitro* studies and in animal models as well as for somatic gene therapy in humans. Following this idea, we designed and made OAT retrovirus vectors that would provide optimal gene expression in deficient human somatic cells

and used this gene delivery system to evaluate OAT expression *in vitro* in an OAT-deficient cell line.

In our previous studies, we were able to express the hOAT gene in murine embryonal fibroblasts. With the aim of achieving a higher expression of hOAT, we analyzed several recombinant retroviral vectors. Although all OAT retrovirus constructs present equivalent levels of functional OAT protein, the enzymatic activity was particularly higher with retrovirus, which was used to transduce an OAT-deficient cell line. Expression of the provirus was studied in the transduced C9 cells, both the 5′ LTR and the internal PGK promoters were active, giving rise to two RNA transcripts. Enzymatic activity in this transduced deficient cell line was at least three times higher over that of hOAT in normal human fibroblasts. However, in GA patients the OAT residual activity is about 5 percent the normal level (5 nmol Δ^1P5C formed/mg/h); therefore, the obtained result of enzymatic activity in the transduced cells (65 nmol Δ^1P5C formed/mg/h) would correspond to more than a 10-fold increase as compared with residual activity in GA patients. Therefore, the level of OAT expression by the LPOSN retrovirus in the *in vitro* system is in the physiologic range needed for the correction of congenital OAT deficiency.

In GA patients, OAT deficiency results in retinal and choroidal degeneration, indicating that OAT function is mostly needed in the eye. This raises the question about the target tissue for somatic gene delivery for gene therapy of this ocular genetic disease. Obviously, the best tissue would be the retinal pigmented epithilium; however, these cells are not surgically accessible for removal, manipulation, and transplantation into the eye of GA patients. In addition, when these cells were isolated from rat or human eyes after biopsy, they were very difficult to maintain in culture. OAT is also highly expressed in liver, where it plays an important role in ornithine metabolism; therefore, hepatic cells could be considered for OAT gene delivery to GA patients. These cells have been previously used successfully by others to correct inborn genetic deficiency in animal models and in humans using both adenovirus and retrovirus. Although both systems have their limitations, the adenovirus gene transfer remains an additional alternative worth pursuing for OAT gene therapy.

There are currently more than 40 approved human gene therapy protocols; nevertheless, to date none of these trials involves ocular diseases. Our data provide the basic knowledge necessary to focus on the appropriate human cells as a target tissue for a future gene therapy trial in this ocular genetic disease.

Proposed Course

Our future efforts will focus on the following issues:

(1) We are correcting the enzymatic activity in cell lines from GA patients.

(2) We will assess the enzymatic activity *in vivo* in animal models after tissue engraft.

(3) We will use the deficient animal that we are creating to assess the feasibility of gene therapy in ocular genetic disease.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Lacorazza DH, Rivero JL, Jendoubi M: Expression of the human OAT gene after retroviral transfer into CHO deficient cell line. *NIH, Research Festival*, EO5:35, 1993.

Lacorazza DH, Jendoubi M: Correction of OAT deficiency in chinesehamster ovary cell line mediated by retrovirus gene transfer. *Sixth International Symposium on Immunology and Opthalmology*, NIH, June 22, 1994:25.

Lacorazza DH, Jendoubi M: Correction of genetic and enzymatic activity of ornithine δ-aminotransferase into mammalian deficient cell lines using retrovirus mediated gene transfer. *ARVO Annual Meeting,* Sarasota, Florida, May 1-6, 1994: 1705.

Lacorazza DH, Rivero LJ, Jendoubi M: Genetic and enzymatic correction of ornithine δ-aminotransferase into CHO deficient cell line. *Gene Therapy,* in press.

Rivero LJ, Laccorazza DH, Kozhigh A, Nussenblatt RB, Jendoubi M: Retrovirus-mediated gene transfer and expression of human ornithine delta transferase into embryonic fibroblasts: An alternative approach to somatic gene therapy. *Hum Gen Ther* 5:701, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00295-01 LI

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Isolation and Characterization of the Mouse OAT Gene for Gene Targeting

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Moncef Jendoubi	Ph.D.	Visiting Scientist	LI, NEI
Others:	Noriko Esumi	M.D., Ph.D.	Visiting Associate	LI, NEI
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	Luis J. Rivero	Ph.D.	Visiting Fellow	LI, NEI

COOPERATING UNITS (if any)

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CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Gyrate atrophy (GA) of the choroid and retina is an autosomal recessive eye disorder involving a progressive loss of vision due to chorioretinal degeneration. A variety of ornithine-δ-aminotransferase (OAT) gene mutations have been reported in GA patients and suspected to associate with this ocular disease. However, the precise mechanism by which the OAT deficiency and hyperornithinemia lead to the chorioretinal degeneration remains unknown. To elucidate the pathophysiological role of OAT, we are attempting to create OAT-deficient mice by gene targeting via embryonic stem (ES) cells. Toward this ultimate goal, we isolated murine OAT gene to construct OAT targeting replacement vector.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

Isolation of Mouse OAT Functional Gene. The 129 mouse genomic library, OLA 129-γGEM-12, was kindly provided by Dr. Anton Berns from the Cancer Institute, Netherlands. This library was screened with a nearly full-length rat ornithine-δ-aminotransferase (OAT) complementary deoxyribonucleic acid (cDNA) probe [4] labeled with ^{32}P-dCTP by random oligonucleotide priming. Screening procedures were performed using standard protocols.

Methods

Polymerase Chain Reaction (PCR) Analysis. To determine the structure of positive genomic clones, PCR was performed with primers from each exon based on mouse cDNA sequence (Genebank, X64837) to amplify each exon and intron. Standard reaction conditions were used.

Sequencing. The promising Clone 11 was subcloned into BlueScript vector (Stratagene, La Jolla, California) and designated pBSmOAT6. This plasmid was sequenced by dideoxy nucleotide chain termination method of Sanger using ^{35}S-dATP and the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs, Beverly, Massachusetts).

Construction of Targeting Vector. The mouse genomic fragment in pBSmOAT6 was 16 kb in length and used almost entirely to make a targeting vector. Neomycin resistance gene (Neo^r) was purified from pMC1neo PolyA kindly provided by Dr. Mario Capecchi and inserted at Sca I site of exon 4 to disrupt the OAT gene. Herpes simplex virus thymidine kinase (HSV-TK) gene was purified from TGV-TK$_2$ (Moncef Jendoubi, unpublished) originated from pIC19R/MC1-TK provided by Dr. Capecchi and added at the 3′ end of the OAT fragment in targeting construct. Standard procedures were used in all subcloning and constructing processes.

Major Findings

Isolation and Characterization of Mouse OAT Gene. By genomic library screening, 16 clones were isolated from 6×10^6 phage plaques. All clones were analyzed by PCR, and the results were compared with the PCR amplification pattern with mouse genomic DNA as a template. Among 16 clones, Clone 11 seemed to encode the functional OAT gene containing the 5′ flanking region and the coding region up to exon 5.

Because the OAT gene has been reported to have at least several pseudogenes and related sequences in human and rat genome [11-14], it is highly suspected that mouse genome also has at least several related sequences. Therefore, sequencing of Clone 11 was performed to confirm that this clone encoded the functional OAT gene. Sequences of a total of 444 bases from exons 3, 4, and 5 were comparable to the sequence of the mouse OAT cDNA.

Construction of Targeting Vector. Targeting vector was constructed according to a positive-negative selection strategy. Using 16 kb fragment in pBSmOATG, exon 4 was disrupted by insertion of Neo^r gene at Sca I site for positive selection by G418. Two targeting vectors were made in which Neo^r gene was introduced in a forward and an opposite direction as referred to the coding strand of OAT gene. HSV-TK gene was added at the 3′ end of the genomic fragment for negative selection by ganciclovir. The length of homologous region was 14 kb upstream from Neo^r and 2.2 kb downstream from Neo^r. This construct is designed for three favorable features: that genomic fragment used is isogenic to ES cell (129 strains of mice), that a positive-negative selection can be used for identifying clones after electroporation into ES cells, and that a long homologous region is expected to yield a high frequency of homologous recombination events.

We made several electroporations using different ES cells for their capacity to contribute to germ-line transmission. Cells were electroporated with OAT targeting construct replacement vector and selected with both G418 and ganciclovir, to enrich the homologous recombination events. Two weeks later, resistant clones were picked up individually and grown for further analysis. Genomic DNA was purified from all clones and analyzed by Southern blot using different restriction digests and differents probes. The results showed that several clones were recombinants on one allele. Four of them already have been injected to generate deficient mice for OAT gene. Presently we are collecting the first litters of chimeric mice for the OAT.

Significance to Biomedical Research and the Program of the Institute

The identification, characterization, mapping, and sequencing of the OAT gene is a very significant achievement in itself because we were able to study the functional OAT gene as well as several pseudogenes.

We used 16 kb to construct two targeting vectors to mutate the functional OAT gene in ES cells. We were able to obtain 17 targeted recombinant clones, four of them have been already injected into embryos to generate deficient animals for the OAT gene. These deficient animals will be invaluable in many respects:

(1) This will be the first animal model for ocular genetic diseases.

(2) We will be able to study the physiological relevance of the OAT gene *in vivo.*

(3) We will also find out if there is any relationship between retina degeneration and the absence of functional OAT enzyme.

Proposed Course

Our work in the future will focus on the following:

(1) We will study the ocular physiology in the OAT deficient mice.

(2) We will assess the importance of the absence of a functional OAT enzyme *in vivo.*

(3) We will use this animal model to assess the feasibility of gene therapy for gyrate atrophy disease.

(4) We will study how to correct the enzymatic deficiency *in vivo* by transferring cells from normal donors to affected animals.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Esumi N, Jendoubi M: Isolation and characterization of the mouse ornithine δ-aminotransferase gene for gene targeting by homologous recombination. *Sixth International Symposium of the Immunology and Immunopathology of the Eye,* 1994, p 25.

Esumi N, Jendoubi M: *Advances in Ocular Immunology.* Amsterdam, Netherlands, Elsevier Press, p. 151, 1994.

Esumi N, Jendoubi M: Isolation, characterization and sequencing of the mouse ornithine δ-aminotransferase gene for gene, in preparation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00288-02 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Gene Therapy for Ocular Genetic Disease

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Moncef Jendoubi	Ph.D.	Visiting Scientist	LI, NEI
Others:	Noriko Esumi	M.D., Ph.D.	Visiting Associate	LI, NEI
	Daniel H. Lacorazza	Ph.D.	Visiting Fellow	LI, NEI
	Luis J. Rivero	Ph.D.	Visiting Fellow	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI

COOPERATING UNITS (*if any*)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section of Genetics and Molecular Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

THIS PROJECT HAS BEEN TERMINATED.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00241-07 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunopathology of Ocular Diseases in Humans

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Chi-Chao Chan	M.D.	Chief, Section on Immunopathology	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Qian Li	M.D.	Visiting Fellow	LI, NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Raymond DeBarge	M.D.	Senior Staff Fellow	LI, NEI
	Scott M. Whitcup	M.D.	Staff Medical Officer	LI, NEI
	Juan Lopez	M.D.	Visiting Associate	LI, NEI
	Miguel Burnier	M.D.	Visiting Scientist	LI, NEI
	Richard Fenton	M.D.	Staff Fellow	LI, NEI
	Dev Shah	M.D.	Visiting Associate	LI, NEI

COOPERATING UNITS *(if any)*

Department of Ophthalmology, Armed Forces Institute of Pathology (Ian W. McLean, M.D.); University of Minnesota, Department of Ophthalmology (Edward J. Holland, M.D.); L'Hôpital de la Pitié, Paris, France (Phuc LeHoang, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated and combined with Project No. Z01 EY 00222-08 LI.

PHS 6040 (Rev. 5/92)

LABORATORY OF MECHANISMS OF OCULAR DISEASES

Report of the Chief
Laboratory of Mechanisms of Ocular Diseases

J. Samuel Zigler, Jr., Ph.D.

Investigators in the Laboratory of Mechanisms of Ocular Diseases (LMOD) are engaged in a broad range of studies relating to the biology of various tissues in the normal eye and the molecular mechanisms responsible for certain ocular diseases. Major emphasis has been on cataract and the various ocular complications of diabetes.

The addition of Dr. Fred Bettelheim to the group as a part-time Special Volunteer and the initiation of new collaborative studies with Dr. Joseph Horwitz, from the Jules Stein Eye Institute, has broadened and strengthened our expertise in the biophysical aspects of lens opacification and in the use of human cataract samples in the laboratory.

Section on Cataracts

Dr. Donita Garland has developed new methods for dissecting the human lens into distinct zones representing tissue produced during different stages of life. Two-dimensional analysis of the proteins present in each of these regions provides a greatly improved picture of the pattern of protein changes that occur as a function of aging and development in the lens. Marked differences are found between the nuclear and cortical protein patterns in both normal lenses and cataracts. Careful analysis of the protein content of dissected zones from intracapsular cataract lenses may provide the most definitive insight to date on the significance, with respect to cataractogenesis, of the changes that occur in both the lens crystallins and the metabolic proteins of the lens.

Dr. Paul Russell and his group are using a variety of model systems, *e.g.*, whole animal, lens organ culture, and lens epithelial cell culture, to investigate the mechanisms that the lens uses to resist environmental and systemic stresses, which can lead to cataract. A great improvement in the efficiency of lens organ culture has resulted from the development of a simple and very effective means to identify lenses damaged during dissection. This allows elimination of bad lenses before experiments begin, thereby greatly reducing the level of variability within experimental groups.

Two new projects have been started during the past year. In one, transgenic mice that develop cataracts as the result of insertion of the human immunodeficiency virus protease gene will be studied to determine how expression of this foreign protease in the lens leads to cataract. In the second new project, serum samples collected from patients with cataract and from normal volunteers will be analyzed for presence of autoantibodies in human lens proteins. By using two-dimensional electrophoresis, the specific protein(s) to which antibodies have been produced can be identified and correlated with cataract type and progression rate.

Dr. Fielding Hejtmancik and his group have had outstanding success in gene linkage studies on several ocular diseases. The locus for Usher's syndrome type I, which they previously linked to chromosome 11q, continues to be refined using fine linkage mapping with the ultimate goal of identifying and characterizing the gene responsible for this disease. Linkage has also been obtained for two different cataract families during the past year. With the establishment of collaborative arrangements with clinical researchers in a variety of locations, including India, Italy, and Barbados, this group will have access to excellent material for the study of inherited cataract, retinal diseases, and glaucoma.

Dr. Deborah Carper's laboratory has continued to study the role of the polyol pathway in the generation of diabetic complications. Site-directed mutagenesis studies on aldose reductase (AR) have identified certain critical residues, information that will be invaluable in the effort to design more effective inhibitors of this enzyme. Studies have also continued on sorbitol dehydrogenase, the second enzyme of the pathway, with determination of the complete structure of the human gene as well as its chromosomal localization. Work is under way to identify the defect in this gene in a family that has cataracts associated with sorbitol dehydrogenase deficiency.

In Dr. J. Samuel Zigler's laboratory, work has concentrated on the testing of potential anticataract agents and on the analysis of the functions of lens crystallins and the role(s) they play in the normal lens and in the process of cataractogenesis. Studies done in collaboration with Dr. Joseph Horwitz are aimed at elucidating the physiological significance of the chaperone-like activity of α-crystallin. The possibility that the "enzyme/crystallins" may be part of the lens' defenses against oxidation is being actively investigated. Studies on the effect of smoke on the lens have provided further support for the view that it represents an important risk factor for cataract development.

Section of Pathophysiology

Dr. W. Gerald Robison and his colleagues are studying the process of diabetic retinopathy. By using advanced morphological and morphometric techniques, they have analyzed the appearance of a variety of specific lesions in a rat model of this disease that are characteristic of changes seen in the human disease. They have also demonstrated that in the rat, these changes can be prevented by AR inhibitors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00105-15 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and Composition of Lens Crystallins With Respect to Cataractogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Samuel Zigler, Jr.	Ph.D.	Research Biologist	LMOD, NEI
Others:	Vasantha Rao	Ph.D.	Visiting Associate	LMOD, NEI
	Pedro Gonzalez	Ph.D.	Visiting Associate	LMOD, NEI
	Chuan Qin	M.D.	Visiting Fellow	LMOD, NEI
	Frederick A. Bettelheim	Ph.D.	Special Volunteer	LMOD, NEI

COOPERATING UNITS *(if any)*

Jules Stein Eye Institute, UCLA (J. Horwitz, Ph.D. and B. Bateman, M.D.); University of Tennessee (H.M. Jernigan, Jr., Ph.D.) National Cancer Institute (M. Krishna, Ph.D.); Centre for Cellular and Molecular Biology, Hyderabad, India (D. Balasubramanian, Ph.D. and M. Rao, Ph.D.)

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project, directed toward elucidation of the molecular mechanisms responsible for cataractogenesis and the development of means of prevention of this disease, places special emphasis on the structure and function of the lens crystallins and the role these proteins play in lens opacification. Until recently, crystallins were thought to be simply structural elements of the lens matrix without specific quantifiable biological functions. Two recent discoveries have provided new insights and approaches to the physiological roles of the crystallins: (1) the crystallins are either functionally active enzymes or are at least related to proteins with specific biological activities, and (2) α-crystallin is a molecular chaperone that can prevent the aggregation of denaturing proteins.

We believe that crystallins have specific biological functions in addition to their structural role in forming the transparent lens tissue. The strongest example to date is the chaperone-like function of α-crystallin. This major lens protein prevents the aggregation of other proteins that are undergoing modification and denaturation. In the lens, where protein turnover is extremely limited and where the long-lived crystallins are known to undergo extensive structural modification, α-crystallin may be essential in preventing protein aggregation and precipitation. Such precipitation would destroy the optical transparency of the lens by creating light scattering centers. We have also developed evidence that the enzyme/crystallins are contributing to the antioxidative capacity of the lens, primarily by markedly increasing the pool of reduced pyridine nucleotides in the lens. We can demonstrate the utilization of the nucleotide's reducing capacity in eliminating activated species of oxygen generated by Fenton chemistry or by other mechanisms. Further support for the view that enzyme/crystallins have specific functions was obtained from studies on the expression of ζ-crystallin in the lenses of guinea pigs and llamas. The data clearly demonstrate that the ζ-crystallin gene was recruited by the lens independently in each of the two species. This finding strongly supports a selective basis for the recruitment rather than a neutral evolution mechanism and indicates that the protein must provide significant benefit to the lens of these species.

Project Description

Objectives

The primary objectives of this project are to: (1) elucidate processes responsible for cataract development at the molecular level, (2) investigate the structures and functions of the lens crystallins, and (3) develop and use model systems for screening potential anticataract agents and test them in appropriate *in vitro* and animal model systems.

Methods

Conventional protein chemical techniques used are chromatography, electrophoresis, and isoelectrofocusing. Immunological studies of lens proteins use specific antisera. Physicochemical analyses on the proteins are performed using high-pressure liquid chromatography, fluorescence, and circular dichroism techniques. Lens organ culture experiments use rat or monkey lenses and use active transport and membrane permeability parameters to monitor the effects of various stresses on the cultured lenses.

Techniques used in analysis of nucleic acids include ribonucleic acid and deoxyribonucleic acid (DNA) isolation, complementary DNA and gene cloning, DNA sequencing, various electrophoretic methods, and the polymerase chain reaction.

Major Findings

(1) In collaboration with Dr. Joseph Horwitz, from the Jules Stein Eye Institute, we have further characterized the chaperone-like activity of α-crystallin and analyzed the specificity of the reaction with different target proteins. With some denaturing proteins, the presence of obligate cofactors is critical if α-crystallin is to effectively prevent aggregation. Circular dichroism spectroscopy suggests that subtle conformational changes associated with the binding of the cofactor may be essential for interaction of α-crystallin with these proteins. Our findings suggest that α-crystallin is specifically adapted to function as a chaperone within the lens.

(2) The putative promoter region previously identified in the llama ζ-crystallin gene has proved to be active by functional analysis. The studies in transfected cells also indicate that the promoter is highly lens specific, *i.e.*, does not drive gene expression in other tissues.

(3) The human gene for ζ-crystallin has been characterized and shown to have essentially the same structure as found in the guinea pig and llama. However, unlike the guinea pig and llama genes, it lacks a second lens-specific promoter and consequently is not expressed at a high level in the lens. A processed pseudogene for ζ-crystallin was also found to be present in the human genome.

(4) A catalytic activity that uses the reducing equivalents of pyridine nucleotides to eliminate free radical oxidants has been identified in the lens. We believe that this activity, which appears to be associated with a protein, may be an important part of the lens' antioxidant capacity. Because NAD(P)H is renewable via redox cycling, it is conceivable that its reducing equivalents may function in a manner analogous to the glutathione redox cycle.

(5) Smoke has been identified as a risk factor for cataract in several epidemiological studies. In collaboration with Dr. Ch. Mohan Rao, from the Center for Cellular and Molecular Biology, Hyderabad, India, we have exposed organ-cultured rat lenses to a condensate of wood smoke. The studies indicate that components of the condensate accumulate within the lens, are metabolized, and cause damage to the cell membranes. Histological analysis demonstrated early and severe injury to the lens epithelial cells.

(6) Studies with Dr. Fred Bettelheim, also using rat lenses in organ culture, have established a calcium-induced cataract model as a system for studying the role of optical anisotropy fluctuations in lens opacification. Early results suggest a major role for the intermedi-

ate filament protein, vimentin, in establishing and maintaining proper order within the cytoplasm of lens fibers. Loss of vimentin via calcium-induced proteolysis leads to optical anisotropy fluctuations that cause lens turbidity.

(7) The testing of potential agents for the prevention or retardation of cataract development is being performed in the lens organ culture system on compounds with antioxidative capacity. Compounds that provide promising results will be prepared for testing *in vivo*.

Significance to Biomedical Research and the Program of the Institute

Cataract is a major public health problem worldwide. Better understanding of the biochemistry of the normal lens and of the molecular changes that occur during aging and cataract development are essential if this disease is to be controlled. Our studies are aimed primarily at elucidating the role of the lens crystallins, the primary structural elements of the normally transparent lens matrix, in the processes leading to opacification. Such knowledge should contribute to the development of means of intervention that can prevent or delay the process of cataract development.

Proposed Course

We will: (1) work to establish viable model systems for testing anticataract agents and use these systems to assess the efficacy of various types of compounds, including antioxidants; (2) clarify the mechanism and the significance of the pyridine nucleotide-dependent antioxidant system in the lens and investigate the possible role of enzyme/crystallins in this system; (3) continue to investigate the chaperone-like function of α-crystallin and determine its physiological significance in the normal lens and in cataract; and (4) expand the two-dimensional analysis of proteins from human normal lenses and cataracts through the use of preliminary fractionation by affinity chromatography. Studies under way using blue Sepharose affinity columns suggest that this will be a very useful way to reduce the complexity of the protein mixture as well as to focus on specific classes of proteins.

NEI Research Program

Lens and Cataract—Pathogenesis of Cataract

Publications

Bettelheim FA, Qin C, Zigler JS Jr: Calcium cataract: A model for optical anisotropy fluctuations. *Exp Eye Res*, in press.

Cui X-L, Qin C, Zigler JS Jr: Residual EDTA bound by lens crystallins accounts for their reported resistance to copper-catalyzed oxidative damage. Arch Biochem Biophys 308:207-213, 1994.

Gonzalez P, Hernandez-Calzadilla D, Rao PV, Rodriquez IR, Zigler JS Jr, Borras T: Comparative analysis of the ζ-crystallin/quinone reductase gene in guinea pig and mouse. Molec Biol Evolution, 11:305-315, 1994.

Gonzalez P, Rao PV, Zigler JS Jr: Organization of the human ζ-crystallin/quinone reductase gene (CRYZ). Genomics 21:317-324, 1994.

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Persson B, Zigler JS Jr, Jornvall H: A superfamily (MOR) of medium chain dehydrogenases/reductases: Sublines including ζ-crystallin, alcohol and polyol dehydrogenases, quinone oxidoreductases, enoyl reductases, VAT-1 and further proteins. Eur J Biochem, in press.

Rao PV, Horwitz J, Zigler JS Jr: Chaperone-like activity of α-crystallin. J Biol Chem 269:13266-13272, 1994.

Tumminia SJ, Qin C, Zigler JS Jr, Russell P: The integrity of mammalian lenses in organ culture. Exp Eye Res 58:367-374, 1994.

Tumminia SJ, Rao Pv, Zigler JS Jr, Russell P: Xenobiotic induction of quinone oxidoreductase activity in lens epithelial cells. Biochim Biophys Acta, 203:251-259, 1993.

Zigler JS Jr: Lens proteins, in Albert DM, Jakobiec F (eds): *Principles and Practice of Ophthalmology.* Basic Sciences, Philadelphia, JB Saunders Co., 1994, pp 97-113.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00189-11 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Oxidation of Proteins in Cataractogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Donita L. Garland	Ph.D.	Research Chemist	LMOD, NEI
Others:	Jose Jimenez	Ph.D.	Visiting Fellow	LMOD, NEI
	Lorenzo Merola	M.S.	Chemist	LMOD, NEI
	Kenichi Matsuno	Ph.D.	IRTA	LMOD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.3	3.3	0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Oxidative processes are a major contributing factor in senile cataracts. We have demonstrated that metal catalyzed oxidation of the crystallins induces protein modifications that mimic those seen in aging, senile cataracts, and brunescent lenses. The importance of the role of metal in oxidative processes related to cataract is further supported by studies in other labs. The lens contains high levels of thiols such as glutathione that can participate in these metal-catalyzed oxidation reactions. Our lab has continued our studies on both the interaction of metals with crystallins and the mechanisms that protect the lens against deleterious oxidation reactions.

A protein that protects enzymes specifically against inactivation by thiol-dependent metal-catalyzed reactions has been reported in yeast and rat tissues. (This activity is not related to catalase, glutathione peroxidase, or superoxide dismutase.) Our lab demonstrated the presence of a similar activity in lenses of bovine, guinea pig, human, pig, monkey, and rat. The antioxidant activity consistently copurifies with a subpopulation of glutathione S-transferase μ.

Copper, zinc, and iron, but not calcium, induced aggregate formation in bovine lens extracts and solutions of the crystallins. Aggregation, measured by light scattering, was time dependent, occurring at metal-to-protein ratios greater than one and varying, depending on the metal and protein. Zinc induced the aggregation of β- and α- but not γ-crystallin. The affinity of copper and zinc for these proteins is relatively low. The addition of EDTA, DETAPAC, L-histidine, or L-cysteine prevented zinc- and copper-induced protein aggregation and caused complete disaggregation. The treatment of α- and β-crystallin and trypsin inhibitor with diethylpyrocarbonate prevented aggregation induced by zinc but not by copper. The presence of salt decreased the metal-induced aggregation of α- and β-crystallin. No metal-induced changes in secondary and tertiary structures of these proteins were observed by fluorescence and circular dichroism spectroscopy.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The long-term goal of this project is to understand the role of oxidation in cataract formation. The immediate objectives are to: (1) study the effect of oxidation on structure and function of lens crystallins, (2) study the interaction between crystallins and those metals that are involved in oxidation/reduction reactions and the effects of these interactions on crystallin solubility and aggregate formation, and (3) characterize the enzymes that protect lens proteins against thiol-dependent metal-catalyzed oxidation.

Methods

Bovine, rat, and guinea pig tissues were used for these studies. We used classical methods to purify proteins. Other methods used were standard procedures for studying proteins, polyacrylamide gel electrophoresis, high-pressure liquid chromatography, ultraviolet spectroscopy, fluorescence, circular dichroism, electron spin resonance, amino acid analysis, immunotechniques, and capillary gas liquid chromatography.

Major Findings

Oxidative processes are considered to be a major contributing factor in senile cataracts. We have demonstrated that metal-catalyzed oxidation of the crystallins induces protein modifications that mimic those seen in aging, senile cataracts, and brunescent lenses. The importance of the role of metal in oxidative processes related to cataract is further supported by studies in other laboratories. The lens contains high levels of thiols such as glutathione that can participate in these metal-catalyzed oxidation reactions. Our laboratory has continued studies on both the interaction of metals with crystallins and the mechanisms that protect the lens against deleterious oxidation reactions.

(1) A protein that protects enzymes specifically against inactivation by thiol-dependent, metal-catalyzed reactions has been reported in yeast and rat tissues. (This activity is not related to catalase, glutathione peroxidase, or superoxide dismutase.) Our laboratory demonstrated the presence of a similar activity in the lens of bovine, guinea pig, human, pig, monkey, and rat. Following the antioxidant activity, a protein was purified from bovine lenses. Seventy percent of the protein sequence was obtained. The protein was identified as glutathione S-transferase μ, a detoxification enzyme found in most cells. This enzyme is not known to possess an antioxidant activity. The antioxidant activity consistently purifies with a subpopulation of glutathione S-transferase through many different purification schemes, yet we have not proved that the antioxidant activity is part of the glutathione S-transferase molecule. It is possible that this subunit may form a heterodimer with glutathione S-transferase. Studies are in progress to try to resolve this issue.

Other lines of evidence indicate the presence in the lens of a protein(s) more closely related to the antioxidant protein characterized from yeast and rat brain. Antibodies made against the whole protein or to an internal peptide crossreact with proteins from lens but not glutathione S-transferase. Further Northern-blot analysis of total ribonucleic acid (RNA) from cow, human, monkey, and rat lens was performed using a complementary deoxyribonucleic acid (cDNA) probe made to the rat antioxidant protein. The results clearly demonstrated the presence of specific messenger RNA for this particular gene.

(2) Copper, zinc, and iron, but not calcium, induced aggregate formation in bovine lens extracts and solutions of the crystallins. Aggregation, measured by light scattering, was time dependent, occurring at metal-to-protein ratios greater than one and varying depending on the metal and protein. Zinc induced the aggregation of β- and α- but not γ-crystallin. Copper and zinc induced the aggregation of a number of other proteins, but they had no effect on lysozyme and papain.

One explanation for the lack of effect on these two proteins is that they are basic proteins. However, copper induced the aggregation of γ-crystallin, also a basic protein.

The affinity of copper and zinc for these proteins is relatively low. The addition of EDTA, DETAPAC, L-histidine, or L-cysteine prevented zinc- and copper-induced protein aggregation and caused complete disaggregation. The treatment of α- and β-crystallin and trypsin inhibitor with diethylpyrocarbonate prevented aggregation induced by zinc but not by copper. The presence of salt decreased the metal-induced aggregation of α- and β-crystallin. No metal-induced changes in secondary and tertiary structures of these proteins were observed by fluorescence and circular dichroism spectroscopy.

In the presence of zinc, lens crystallins were least soluble at 2.0 - 2.5 pH units higher than their isoelectric points. The effects of varying pH were fully reversible. Solubility decreased further with increasing temperature. Lowering the temperature did not induce any disaggregation, indicating that the temperature effect on zinc-induced aggregation was not reversible.

The mechanism of metal-induced aggregation is not clear. Effects of the metals on conformation could not be demonstrated. The data obtained to date support a neutralization mechanism of aggregation more than a cross-linking mechanism, but these studies are still in progress.

(3) Capillary gas chromatography (GLC) is used in this laboratory to study sugar metabolism in the lens of two animal models, rat and monkey. Standard methodologies have been used to quantitate the common sugars. Methods have been devised in this laboratory that allow the separation of sorbitol and dulcitol, the alcohol derivatives of glucose and galactose. Sugar analyses have been done on serum of animals fed various sugar diets and rat lenses incubated *in vitro* with a variety of sugars.

The chromatogram obtained from the capillary GLC analysis of animal blood serums and lens material contains, in addition to the expected sugar derivatives, a number of unidentified metabolites present in low quantities. Our laboratory is in the process of identifying these. One of those metabolites present in both rat and monkey lenses, has been tentatively identified as scylloinositol.

Significance to Biomedical Research and the Program of the Institute

Oxidative processes are known to contribute to cataractogensis. Metal-catalyzed oxidation of the crystallins leads to protein modification that mimic those seen in aging, senile cataracts, and brunescent lenses. Studies on the interaction of metals with the crystallins are highly relevant to understanding the role of these oxidation reactions in lens. Understanding those mechanisms present in lens for protecting against oxidative damage is important for developing interventions. Studies on glutathione S-transferase are highly relevant to cataract. Recent reports correlate deletions on glutathione S-transferase μ with certain cataracts.

Proposed Course

We will focus our studies for fiscal year 1995 on the following: (1) pursuing the activity in lens that protects against thiol-dependent oxidation reactions, (2) assaying for glutathione S-transferases in epithelial layers from cataract surgeries, and (3) continuing the studies on the interaction of metals with the crystallins.

NEI Research Program

Lens and Cataract—Pathogenesis of Cataract

Publications

Bettelheim FA, Reid MB, Garland D: Hydration of gamma crystallins. *Exp Eye Res* 58:219-224, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00296-01 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on Human Lens Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Donita L. Garland	Ph.D.	Research Chemist	LMOD, NEI
Others:	J. Samuel Zigler, Jr.	Ph.D.	Senior Scientist	LMOD, NEI
	Yvonne Duglas-Tabor	B.A./B.S.	Biologist	OGCS, NEI

COOPERATING UNITS *(if any)*

Ophthalmic Genetics and Clinical Services Branch, NEI, NIH (Manuel B. Datiles, M.D.); Jules Stein Eye Institute, UCLA (J. Horwitz, Ph.D.)

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This laboratory has in progress a study to characterize the proteins of the human lens. The lens consists of a few very high abundance proteins called the crystallins and several hundred lesser abundance proteins. During the life of the lens, the proteins become extensively modified. Methodologies have been developed to yield good separation of the proteins using two-dimensional polyacrylamide gel electrophoresis. Due to the extensive modifications, identification of the proteins is based on immunotechniques and sequencing. Normal donor lenses varying in age from fetal to 70 years and cataracts of different etiologies have been analyzed. The regions of the normal lenses are identified by structure patterns. The cortex has been separated into three different layers, and each of the developmentally defined nuclear regions has been separated. The protein patterns in each of the cortical regions are distinguishable as cortex with the characteristic large protein spots corresponding to each of the major crystallins. The protein patterns of the nuclear regions are all similar to each other but clearly unique from those of the cortical regions. Protein spots corresponding to the major crystallins are not readily visible. Many new spots are present, including numerous low molecular weight spots that are fragments of crystallins. These results have significance in understanding the potential role of protein modification in cataractogenesis. The proteins throughout the lens are being identified, yielding a database of information on the normal human lens. The protein patterns of numerous cataracts have been determined. These data are now being analyzed with respect to the cataract etiology.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The long-term goal of this project is to understand those mechanisms involved in human cataractogenesis.

The immediate objectives of this project are to: (1) identify the proteins of the human lens and characterize the changes in the protein composition that occur with development and aging, (2) identify the covalent modifications that the human lens proteins undergo with development and aging, and (3) characterize the proteins of human cataracts of various etiologies.

Methods

Human lens material was obtained from donors' eyes and from cataract surgery. Immobilized pH gradients and the Isodalt two-dimensional electrophoresis system were used to separate the lens proteins. Other techniques used include high-pressure liquid chromatography, ultraviolet/visible spectroscopy, and immunotechniques.

Major Findings

Normal donor lenses of ages varying from seven months to 60 years were dissected, and the lens regions were identified by suture patterns. Adult lenses were easily and clearly separated into regions corresponding to elongating fiber cells, outer cortex, inner cortex, adult nucleus, fetal nucleus, and embryonic nucleus. In some lenses, another layer was observed that may correspond to the juvenile nucleus. These separations are consistent with the zones of discontinuity seen microscopically *in vivo*.

The proteins of each region were separated by two-dimensional electrophoresis. For the three cortex layers, the two-dimensional protein patterns are distinguishable as cortex. These patterns are characterized by large protein spots corresponding to αA-, αB-, βB1-γS-, and βB2-crystallins as well as 100 to 200 lesser abundant spots. Comparisons of the three cortex regions yield some differences among the protein patterns. The inner cortex of the mature lens has an increase in the acidic forms of α crystallin, bB1-crystallin spots are greatly diminished, and many lesser abundant spots are either increased or decreased.

In contrast, the protein patterns of the nuclear regions of adult lenses are unique and distinguishable from those of the cortical regions. The crystallins have undergone extensive modification. Protein spots corresponding to the major crystallins are not visible. Many new spots are present, and there is a significant increase in the number of low molecular spots that are fragments of crystallins. The protein patterns, including the low molecular weight species, are similar in all adult lenses analyzed. The clear distinction between the protein patterns of the cortex and those of the nucleus suggests that certain modifications of the crystallins occur in the developmentally defined nuclear regions but not in the cortical regions. It is not clear if there are signals that initiate the modifications of crystallins in the nucleus or if there are signals that prevention the modifications of proteins in the cortex.

The protein patterns of the adult, fetal, and embryonic nuclear regions are similar in the adult lens. The protein patterns of the nuclear regions of lenses from birth to about 30 years change with increasing age from a pattern closer to a cortical pattern to that of a nuclear protein pattern. For lenses older than 30 years, the protein patterns of the nuclear regions appear to remain stable.

Predictable protein patterns are found in all normal lenses in comparable regions of lenses of comparable ages. Thus, these results are consistent with the interpretation that the extensive modifications seen in the two-dimensional protein patterns do not represent deleterious, random modifications that result from decreased lens viability or form exposure to noxious environmental agents. These

modifications are likely not related to cataract formation. Rather at appropriate developmental stages, a series of reactions occur leading to the covalent modification of the crystallins. These modified forms of the crystallins may be better suited for function in the center of the lens in maintaining transparency.

Numerous studies have demonstrated that with time many of the lens proteins undergo acidification. Our studies demonstrate that the enzymes such as glyceraldehyde 3-phosphate dehydrogenase are present as multiple-charge species even in the outermost layers of the cortex. This suggests that the conversion of these enzymes to more acidic forms is not likely to be just the result of aging as has been suggested.

The proteins of the cataractous regions of posterior subcapsular cataracts with diagnoses of senile, presenile, radiation, retinitis pigmentosa, and gyrate atrophy have been analyzed by two-dimensional electrophoresis. Some differences in the protein patterns exist, but, so far, no protein changes can be correlated with any disease etiology.

The proteins of a large number of cortical and nuclear cataracts have been analyzed. Correlations between protein changes and cataract types have not yet been found using the present methods of classifying cataracts. The data obtained to date indicate that there are no obvious, unique crystallin modifications responsible for each type of cataract.

Many of the proteins in the human lens have been identified using immunoblotting techniques. Degraded forms of αB-crystallin have been identified in both normal and cataractous lenses.

Blots of human lens proteins separated by two-dimensional electrophoresis are being used to identify the targets of autoantibodies found in normal and cataract patients.

Significance to Biomedical Research and the Program of the Institute

These studies are directly relevant to the NEI program. Characterization of the proteins of the normal human lens will provide information necessary to understand cataractogenesis. In addition, it will provide information on normal metabolic and developmental processes in this unique tissue in contrast to those processes that occur as a function of aging.

Proposed Course

In fiscal year 1995, we will continue the characterization of human lens proteins. Our studies will include continued efforts to identify the modified proteins and the modifications involved, the purification of modified crystallins for sequencing and mass spectroscopy, and the continued analysis of cataracts.

NEI Research Program

Lens and Cataract—Lens Development and Aging

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00201-10 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and Expression of Polyol Pathway Enzymes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Deborah Carper	Ph.D.	Biologist	LMOD, NEI
Others:	Susan Old	Ph.D.	Staff Fellow	LMOD, NEI
	Takeshi Iwata	Ph.D.	Visiting Associate	LMOD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In tissues that do not require insulin for glucose uptake, the high systemic level of glucose that develops during diabetic hyperglycemia readily translates into high tissue levels of glucose. Some of this excess glucose is metabolized by the polyol pathway. Aldose reductase (AR), the first enzyme of this pathway, reduces glucose to the organic osmolyte sorbitol while sorbitol dehydrogenase (SDH), the second enzyme of the pathway, oxidizes sorbitol to fructose. The diabetes-enhanced flux of glucose through the polyol pathway has been implicated in the etiology of diabetic complications, including cataract, retinopathy, and neuropathy. Our studies have been aimed at defining the structure and regulation of AR and SDH so that new approaches to the control of this pathway may be made available in diabetic tissues.

Many aldose reductase inhibitors (ARIs) have been shown to have broad substrate specificity and undesirable side effects. Emphasis on the structure/function properties of the AR enzyme will help in the refinement and design of future inhibitors. To this end, catalysis and inhibition of aldose reductase was examined following site-directed mutagenesis. In the rat, our mutagenesis studies indicated that tyrosine 48, histidine 110, and cysteine 298 are important residues in the active site and that tyrosine 48 is most likely the proton donor during substrate reduction. In addition, mutation of histidine 110, an active-site residue, decreased inhibitor effectiveness by up to 2000-fold, indicating that this residue is important in inhibitor binding. Mutations of human AR indicated that although phenylalanine 122 putatively binds ARIs, substitution of this amino acid has little affect on the inhibitor-binding constant, K_i.

Sorbitol dehydrogenase has been reported to be linked to cataract formation in nondiabetics. We have determined the gene structure, tissue distribution, transcriptional initiation, and chromosomal localization of human SDH. In addition we have used SSCP analysis followed by sequence comparison to analyze congenital cataract patients with SDH deficiency. Through these studies, we hope to identify the gene defect in this family.

Project Description

Objectives

The objective of this project is to study the structure, function, and regulation of the two enzymes of the polyol pathway—aldose reductase (AR) and sorbitol dehydrogenase (SDH).

Methods

The methods used include molecular biology, protein chemistry, cell biology, and molecular genetics.

Major Findings

Structure-function studies of AR. X-ray crystallographic studies previously indicated that either Y48, H110, or C298 could potentially function as the proton donor in the catalytic mechanism of AR. The mechanism involves binding of the substrate to the enzyme/NADPH complex with subsequent hydride transfer from the nicotinamide ring to the carbonyl group. A proton is then donated to the carbonyl oxygen. Our studies showed that mutagenesis of rat lens tyrosine 48 to phenylalanine (Y48F) virtually abolished rat lens enzyme activity, even though the physical structure of Y48F appeared to be normal as evidenced by circular dichroism spectra and NADPH-binding affinity constants, Kd. Changes of histidine 110 to glutamine (H110Q) and cysteine 298 to serine (C298S) resulted in mutants that were still active. Our findings would indicate that Y48 is the proton donor in the reduction reaction of rat lens AR.

The potential role of the amino acids C298, H110, H187, and H200 in the inhibition of rat lens AR was examined by measuring the IC50 values of five different AR inhibitors. No differences between the wild type and the mutants were observed, except for H110Q. This AR mutant was less sensitive to inhibition by both the carboxylic and hydantoin classes of compounds. The greatest increase in IC50 was seen with the hydantoins. There was a three hundredfold increase for sorbinil and a two thousandfold increase for imeristat. The carboxylic acids such as tolrestat and statil gave increases of approximately fivefold. These inhibitor studies indicate that H110 is important in inhibitor binding. The mechanism involved may include perturbation of the positively charged anion well formed by Y48, H110, and the nicotiniamide ring with variable effects on AR inhibitors due to their inherently different structural properties. The efficacy of AR inhibitors are evaluated by their ability to lower polyol levels and prevent or retard cataract formation, retinal microangiopathies, microalbuminuria, decreased motor nerve conduction velocity, and/or deterioration of nerve ultrastructure. Because the rat is the most widely used model of diabetic complications, comparative information on the catalysis and inhibition of rat and human AR has provided a baseline for designing specific inhibitors.

Our studies on the mutagenesis of human AR have been focused on elucidating the residues involved in inhibitor binding. X-ray crystallographic studies indicated that a number of hydrophobic interactions occur between the enzyme and the inhibitor. The inhibitor was proposed to be sequestered by a hydrophobic bridge comprising phenylalanine 122 and leucine 300. We have begun to evaluate this location by mutating phenylalanine 122 to tyrosine and cysteine. Small changes in the affinity to various substrates, Km, and in the turnover number, Kcat, were observed. However, contrary to the predicted importance of this residue, there was no significant change in the inhibitor-binding constant, Ki.

Gene structure of sorbitol dehydrogenase. The complete complementary deoxyribonucleic acid (cDNA) sequence coding for human SDH and the genomic organization of this enzyme were determined. SDH is arranged into nine exons and eight introns. The first exon contains 89 bp of 5′ untranslated sequence, and exon nine contains 1,263 bp of 3′ untranslated sequence. This considerably long stretch of 3′ untranslated sequence comprises more than 60 percent of the total cDNA sequence, the im-

portance of which is unknown, although it is likely to include messenger ribonucleic acid stability and translational regulation. Homologous human alcohol dehydrogenase genes and the human ζ-crystallin gene are also arranged into nine exons and eight introns, but none of the splicing points coincide with the splice points of the SDH gene. At the promoter region of human SDH, no obvious TATAA or CCAAT box was found.

Interestingly, different transcription initiation sites were observed in liver and lens. These different transcription initiation sites do not affect the translation initiation site (ATG-codon). At the 5' flanking region of this gene, which is reported to bind Sp1 transcriptional factor, three Sp1 and a CACCC box were observed. The sequential motif of the promoter region resembles that of duck lactate dehydrogenase β/ε-crystallin that is highly expressed in heart as an enzyme and in lens as a crystallin. That SDH may also be an enzyme/crystallin is an interesting possibility. Fluorescence *in situ* hybridization localized the SDH gene to human chromosome 15q21.1.

Northern blot analysis demonstrated that the highest expression of SDH was in lens. Other tissues with high expression were kidney, heart, brain, testes, retina, and the retinoblastoma cell line Y79. The high expression of SDH in human lens is of great interest in view of previous reports showing abnormal SDH activity in red blood cells of patients who develop cataracts.

A congenital cataract patient from the largest known family with red blood cell SDH enzyme deficiency was examined for mutations by single standard conformation polymorphisms and DNA sequencing. In this family, four out of five brothers and their father had bilateral cataracts. Although SDH activity is reduced 18 to 78 percent of normal in all members of this family, the occurence of cataract does not appear to be correlated with the severity of the red blood cell deficiency. However, this does not rule out a correlation between cataract and lens SDH activity. In this study, a polymorphic one bp mismatch in exon four and a two bp deletion followed by a one bp mismatch in exon nine were found, but no mutation within the coding sequence was detected. Thus, the enzyme deficiency is probably not due to abnormal protein structure, and other possibilities such as reduced promoter activity are now being examined. We have demonstrated high expression of SDH in human lens compared with other tissues. A previous study reported higher enzyme activity in human lens compared with other species. These data suggest that SDH may play an important role in the human lens, and dysfunction of this enzyme may lead to alterations in the polyol pathway.

Significance to Biomedical Research and the Program of the Institute

The polyol pathway has been implicated in ocular and other complications of diabetes. Regulating the enzymes of this pathway—AR and SDH—in a manner that will decrease the flux of glucose through the polyol pathway should ameliorate these complications. Using structure/function studies, the localization of the inhibitor site of AR will provide a rational basis for inhibitor drug design. By characterizing SDH, we can begin to evaluate its role in the lens and its interrelationship with AR.

Proposed Course

Site-directed mutagenesis studies will continue to be used to determine the inhibitor-binding site of human AR. For SDH, the functional promoter will be defined, and the other members of the family with lowered SDH activity and cataracts will be examined.

NEI Research Program

Lens and Cataract—Molecular Genetics

Publications

Bateman JB, Kojis T, Heinzmann C, Klisak I, Diep A, Carper D, Nishimura C, Mohandas T, Sparkes R: Mapping of AR gene sequences to

human chromosomes 1, 3, 7, 9, 11, and 13. *Genomics* 17:560-565, 1993.

Iwata T, Carper D: *Human Sorbitol Dehydrogenase Gene.* New York, Plenum Publishing Corp., in press.

Old SE, Carper DA, Hohman, TC: Na,K-ATPase response to osmotic stress in primary dog lens epithelial cells. *Invest Ophthalmol Vis Sci,* in press.

Sato S, Old S, Carper D, Kador PF: *Purification and Characterization of Recombinant Human Placental and Rat Lens ARs Expressed in* Escherichia coli. New York, Plenum Publishing Corp., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00237-09 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Characterization of the Lens

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Paul Russell	Ph.D.	Research Chemist	LMOD, NEI
Others:	Carolyn Chambers	Ph.D.	Senior Staff Fellow	LMOD, NEI
	Geoffrey Kidd	Ph.D.	Senior Staff Fellow	LMOD, NEI
	Santa Tumminia	Ph.D.	Senior Staff Fellow	LMOD, NEI

COOPERATING UNITS (*if any*)

Oakland University (John Reddan, Ph.D.); Purdue University (Jean Smith, Ph.D.); University of East Anglia (George Duncan, Ph.D.); National Institute of Child Health and Human Development (Jose Pichel, Ph.D. and Heiner Westphal, Ph.D.)

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.6	3.6	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

We are continuing our efforts in characterizing the lens and processes that may occur in cataract development. There are three aims in our research efforts: determination of the seqeuences of human crystallins, development of the *in vitro* lens incubation system, and examination of stress on lenses and lens crystallins. Each of these areas complements the other and builds a base on which to study how the lens resists cataract development and the stresses that will eventually lead of opacification.

The first area of work has been the determination of the sequence of human β B2-crystallin and human γS-crystallin. The human β B2-crystallin is the principal β crystallin in the lens. Two human lens complementary deoxyribonucleic acid (cDNA) libraries were made. One of the libraries was to adult lens and one was to fetal lens. The β B2-crystallin cDNA was cloned and sequenced from these libraries and the deduced amino acid sequence was determined. Work also continued on the promoter region of this crystallin in order to develop a construct that would be developmentally regulated in transgenic animals. γS-crystallin is a protein that increases in content in the lens with age. The amount of this crystallin is very often decreased with the advent of human cataract formation. The γS-crystallin sequence was determined by a combination of cDNA sequencing, electrospray ionization mass spectrometry, and fast atom bombardment mass spectrometry.

In vitro lens incubation is currently one of the only ways to study the effect of environmental stresses on whole lens metabolism. Work has progressed to establish criteria for determining which of the *in vitro* incubated lenses has metabolic integrity and has not been injured in the dissection process. A simple test has been developed to determine the integrity of the lens *in vitro* in as few as 30 minutes after the start of the incubation procedure. Studies have been done *in vitro* to determine the response of the whole lens to oxidative insult using the content and mRNA levels of catalase, an enzyme responsible for protection against H_2O_2. Glutathione levels of stressed lenses have also been measured to determine the reason for the loss of this vital constituent.

The effects of environmental stresses have also been studied using cell culture systems. The cell line used in these studies constitutively expresses αB-crystallin. Effects of oxidative stress and heat shock have been investigated on this crystallin that is thought to serve as a molecular chaperon.

Project Description

Objectives

The purposes of this project are to: (1) determine the components in the human lens, (2) develop model systems to examine how stresses such as oxidation can alter the tissues in the anterior segment of the eye, (3) define these model systems for use with agents that might delay cataract formation, and (4) explore basic questions about the metabolism of the lens using cell and molecular biological methods.

Methods

Among numerous biochemical and molecular biological methods used in this research are Northern, Southern, and Western blotting of messenger ribonucleic acid, deoxyribonucleic acid (DNA), and proteins. In addition, various methods for quantitation of these components such as slot-blotting are done. The polymerase chain reaction is used as is nucleic acid sequencing.

Major Findings

(1) Two libraries of the complementary cDNAs from human lenses have been made. One of the libraries is from adult lens, and one is from fetal lens.

(2) The sequence of the human βB2-crystallin has been determined from cloned sequences from the human lens libraries. The sequence of the human βB2-crystallin is similar to the sequence of the bovine and rat crystallins and shows the high level of homology in this crystallin. The deduced sequence of the βB2-crystallin matches exactly the protein sequence that was published concurrently by others.

(3) The sequence of human γS-crystallin has been determined using a combination of cDNA sequencing, electrospray ionization mass spectrometry, and fast atom bombardment mass spectrometry. The human sequence differs from the bovine sequence in several areas, including one peptide fragment that appears to be associated with cataract development.

(4) A method for the determination of protein content in the incubation medium has been tested as a means to determine lens integrity *in vitro*. The method, which is rapid, can accurately predict which of the lenses *in vitro* has been metabolically compromised during the dissection procedure.

(5) Studies on rat and monkey lenses incubated *in vitro* have suggested that the rapid loss of glutathione in the young rat lenses *in vitro* may be due to the rapid growth rate of these lenses. Older lenses and lenses from adolescent monkeys do not exhibit the rapid loss of this constituent. The loss of glutathione in the rat lens has long been an enigma.

(6) αB-crystallin, a molecular chaperone, has been studied using a cultured cell line that constitutively expresses this protein. In the U373MG astroglioma cell line, the accumulation of this crystallin has been shown to be stress dependent and phosphorylation independent. Heat shock will cause a rapid rise in the level of this protein, but within four to six hours the levels of αB-crystallin return to baseline values. Incubation of the cell with cobalt will also cause a rise but at a later time, and the level does not return to baseline within 72 hours. The αB-crystallin also appears to switch from water soluble to water insoluble after the insult suggesting an alteration in the compartmentalization of this chaperone. The level of phosphorylation of the αB-crystallin was not changed during the stress experiments, indicating phosphorylation was not an important event for the function of this protein in response to stress.

Significance to Biomedical Research and the Program of the Institute

The human lens needs to be characterized to determine which components are involved in the protection of this tissue from environmen-

tal pressures such as oxidative stress and which components are the more susceptible to these stresses. Once the constituents in the human lens are known, the development of systems to study the whole lens is vital to understanding the process of cataract formation. Obtaining a reproducible *in vitro* system is important to the development of anticataract agents. Working under definable conditions will allow us to formulate mechanisms and agents to ameliorate cataract development.

NEI Research Program

Lens and Cataract—Lens Biochemistry and Biophysics

Publications

Chambers C, Russell P: Sequence of the human lens βB2 crystallin encoding cDNA. *Gene* 133:295-299, 1993.

Kidd GL, Reddan JR, Russell P: Differentiation and angiogenic growth factor message in two mammalian lens epithelial cells lines. *Differentiation* 6:67-74, 1994.

Russell P, Tumminia SJ, Pichel JMC: Comparison of lens epithelial cell lines from transgenic animals. *Invest Ophthalmol Vis Sci* 35:2204, 1994.

Tumminia SJ, Qin C, Zigler JS Jr, Russell P: The integrity of mammalian lenses in organ culture. *Exp Eye Res* 58:367-374, 1994.

Tumminia SJ, Russell P: αB-crystallin expression and chaperone function in human astroglioma cell line U373. *Invest Ophthalmol Vis Sci* 35:2212, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00289-01 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Lenticular Expression of the HIV Protease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Paul Russell	Ph.D.	Research Chemist	LMOD, NEI
Others:	Santa Tumminia	Ph.D.	Senior Staff Fellow	LMOD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.35	0.35	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two transgenic mouse strains have recently been obtained. These strains contain the HIV protease coding sequence linked to the αA-crystallin promoter. One strain gets cataract *in utero*, while the other strain develops cataract at approximately 25 days. The strains are currently hemizygous. Efforts in the past several months have been directed to getting homozygous populations of these strains to determine the cause of the cataract formation and how this is related to the expression of the HIV protease in the lens.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The purpose of this project is to determine how the expression of the human immunodeficiency virus protease in the lens causes cataract formation.

Methods

We will use biochemical and molecular biological methods as well as light and electron microscopy to investigate cataract formation.

Major Findings

The homozygous mouse strains appear to get cataract formation a few days before the hemizygous animals; therefore, we are using homozygous selection.

Significance to Biomedical Research and the Program of the Institute

Cataract formation in the presence of a small amount of HIV protease in the lens could suggest that the protease is cleaving a specific protein that will start the process of cataract formation. Alternatively, the protease could be interfering with the process of differentiation in the lens. Either of these two hypotheses would have important implications either for cataract formation or for the action of the HIV virus in general.

Proposed Course

After developing the homozygous strains, we will investigate the mechanism involved in cataract formation in these animals.

NEI Research Program

Lens and Cataract—Pathogenesis of Cataract

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00290-01 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Autoantibodies to Lens Crystallins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Paul Russell	Ph.D.	Research Chemist	LMOD, NEI
Others:	J. Samuel Zigler, Jr.	Ph.D.	Senior Scientist	LMOD, NEI
	Donita Garland	Ph.D.	Research Biologist	LMOD, NEI
	Yvonne Duglas-Tabor	B.A./B.S.	Biologist	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Experiments are under way to investigate the level of autoantibodies to lens crystallins in the serum of normal volunteers and of individuals with cataracts. The specific autoantibodies will be determined and measured using chemiluminescent Western blots of two-dimensional gels of human lens proteins. Initial efforts on some serum samples have indicated that autoantibodies are present, and these appear to react most commonly with the β-crystallin components in the lens.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The purpose of this project is to determine if autoantibodies to lens are present in the serum and if the specific autoantibodies correlate with cataract type or the rate of progression of cataract. We will use chemiluminescent Western blot analysis to investigate the increase in autoantibodies.

Methods

Autoantibodies will be determined using Western blots to lens proteins. The lens proteins will be separated by two-dimensional gel electrophoresis.

Major Findings

This project has just recently been undertaken; however, it has been determined that autoantibodies are present in the serum. The autoantibodies that have been observed are generally to proteins that are part of the β-crystallin family.

Significance to Biomedical Research and the Program of the Institute

This project sets out to determine if specific cataract type or the progression of cataract formation can be related to specific autoantibodies to lens crystallins that are present in the serum. If a correlation between these autoantibodies and progression of lens opacification can be made, it might be possible to develop a clinical test to determine which individuals are most at risk for rapid cataract formation.

Proposed Course

Efforts will continue to determine the level of autoantibodies in the serum of normal individuals, and then these levels will be compared with the ones found in the serum of patients who have cataracts.

NEI Research Program

Lens and Cataract—Pathogenesis of Cataract

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00272-04 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Inherited Ocular Diseases

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	James Fielding Hejtmancik	M.D., Ph.D.	Medical Officer	LMOD, NEI
Others:	John Hope	Ph.D.	Senior Fellow	LMOD, NEI
	Radha Ayyagari	Ph.D.	Visiting Associate	LMOD, NEI
	Ling Lee	M.S.	Chemist	LMOD, NEI
	Masami Oguni	M.D.	Fogarty Fellow	LMOD, NEI
	Rita Mitra	Ph.D.	Special Volunteer	LMOD, NEI
	T. Padma	Ph.D.	Visiting Scientist	LMOD, NEI

COOPERATING UNITS (*if any*)

Baylor College of Medicine (J. Towbin, M.D.); Univ. of Iowa (R. Smith, M.D.); Univ. of Texas-Houston (S. Daiger, Ph.D.); Ocular Genetics and Clinical Services Branch, NEI, NIH (M. Kaiser, M.D., R.Caruso, M.D., R. Sperduto, M.D.); Massachusetts Institute of Technology (G. Benedek, Ph.D., J. Pande, Ph.D.); Osmania Univ., Hyderabad, India (J.S. Murty, Ph.D., T. Padma, Ph.D.); L.V. Prasad Eye Inst., Hyderabad, India (G.N. Rao, M.D., S. Basti, Ph.D.); Universita Degli Studi di Parma (G. Maraini, M.D., G. Alberti, M.D.)

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.67	5.67	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Study of inherited visual diseases provides a means by which both normal and aberrant visual processes might be understood. In addition to directly elucidating the pathophysiology of the inherited disease under study, these studies can provide insights into the structure-function relationships of the molecular components of the visual system and their normal physiology. This laboratory is using a number of approaches to study inherited visual diseases affecting the lens and retina.

Lens crystallins make up more than 90 percent of the soluble protein of the lens and are heavily modified in most cataracts. The effects that specific modifications of β- and γ-crystallin structure produce on crystallin functions such as stability and formation of macromolecular aggregates are being expressed using SF9 cells transformed with bacculovirus vector containing coding sequences for normal and modified β A3/A1- and β A2-crystallin genes. Regions of the β-crystallin molecule of special interest include the amino and carboxy terminal arms, the connecting peptide, and the Greek key motifs of the core domains. In addition, the interactions of acidic and basic β-crystallins are being studied.

A second approach to understanding inherited visual diseases uses principles of positional cloning to identify genes important in human inherited diseases. Human diseases currently undergoing linkage analysis, gene isolation, or characterization of mutations include Usher syndrome, long QT syndrome, cataracts, and a variety of X-linked syndromes. We are currently collecting families with autosomal recessive retinitis pigmentosa and Bietti syndrome in preparation for study of this important group of diseases. Finally, the effects of specific genetic alterations, including red pigment gene polymorphisms and glutathione S-transferase M1 deletions on the visual process, are being studied.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The long-range objectives of this project include increasing the understanding of inherited visual diseases, with the eventual aims of increasing the diagnostic ability for these diseases and providing a foundation for developing rational therapies based on a thorough knowledge of their molecular pathophysiology. These long-range objectives will be approached by pursuing the specific aims of identifying genes involved in inherited visual diseases and elucidating the mechanisms by which mutations in these genes cause disease.

Methods

Conventional cloning technology is used in preparing sequences for gene expression studies. These include ligation with T4 deoxyribonucleic acid (DNA) ligase, screening by NaOH miniprep methodology, and ^{32}P-labeled DNA probes as well as allele-specific oligonucleotide hybridization to screen for specific single-base settings. Sequence changes are introduced by site-specific mutagenesis using standard methodology. Gene expression is carried out in insect cells (Sf9) using the baculovirus expression system. Protein expression is monitored by standard two-dimensional gel electrophoresis followed by immunoblotting. Association behavior is assessed by elution volume on sieve FPLC.

Crystallin and other complementary DNAs and genomic fragments are isolated by library screening with cloned genes or oligonucleotides using routine methods. Sequencing is carried out by cycling or using automated florescent technology.

Until recently, linkage analysis has been carried out by conventional Southern blotting. Cell lines from patients and other family members are immortalized by Epstein Barr virus transformation. DNA is isolated by standard methodology and digested by restriction endonucleases. After agarose gel electrophoresis, Southern transfer is performed and the resulting blot probed with isolated DNA fragments labeled with [^{32}P] by oligonucleotide labeling. Recently, short tandem repeat (STR, microsatellite) markers have been analyzed by polymerase chain reaction performed in the presence of labeled oligonucleotides and analyzed on sequencing gels. Linkage data are recorded on a computerized spreadsheet and analyzed using both two-point and multipoint analysis with the LINKAGE program package.

Major Findings

(1) The β-crystallins, their structure, and the mechanisms by which heterogeneity arises among this family of proteins are being investigated. The βA3-crystallin is identical to βA1 except for an additional 17 amino acid N-terminal extension. The same gene is believed to encode and express both polypeptides. In addition, the βA3/A1 coding sequences were inserted into the Bluebac expression vector (Stratagene) and expressed in SF9 cells. In SF9 cells, a protein with the same amino terminal sequence as the βA1-crystallin is produced when the baculovirus-infected cells are grown past their prime. This is temporally correlated with the disappearance of the βA3-crystallin band, suggesting that the smaller band is created by processing or by degradation of the larger in this system. In addition, clones for the mouse βA2-, βB1-, βB2-, and βB3-crystallins have been isolated and sequenced in preparation for characterization of their roles in β-crystallin aggregation.

(2) An additional crystallin has been constructed in which the amino terminal arm is deleted and replaced by a glycine residue, so that this extension is identical to that found in γ2-crystallin. This has been expressed in SF9 cells (Blubac vector) and has an appropriate migration on Laemli gels, CD-spectrum, and amino acid sequence. The activity of this β-crystallin in association into the typical 200-250 kDa aggregates has been tested using FPLC on superdex 75 and superose columns. The normal βA3 polypeptide readily associ-

ates into homodimers, but the truncated βA3 associates minimally if at all. SF9 cells expressing the recombinant crystallins were grown in $^{[35]}$S containing medium purified and reassociated with an excess of lens extract containing normal crystallins (unlabeled) using limited urea denaturation followed by dialysis. Association into β-crystallin aggregates was assessed by FPLC on sizing columns. The recombinant full-length β-crystallin peptide associates into both dimers and tetramers, with the dimer peak migrating slightly before the β-light peak. The truncated βA3-crystallin, however, migrates slightly behind the β-light peak and does not form obvious tetramers. These data strongly suggest that the amino terminal arm of β-crystallins assists in the association of β-crystallins into higher order aggregates.

(3) A βA3-crystallin has been constructed in which the entire connecting peptide from the first to the second domain has been replaced with the corresponding sequence from γ2-crystallin. This tests the hypothesis that the connecting peptide, which crystallographic data show is extended in the β-crystallins and curved back on itself in the γ-crystallins, is responsible in this fashion for the β-crystallins' tendency to dimerize. The β-crystallin with the modified connecting peptide was subjected to the same tests of association as described in number 2 and behaved essentially as the normal (unmodified) βA3-crystallin. The secondary structure of the modified β-crystallin is currently being confirmed with CD analysis.

(4) The mouse βB2-crystallin has been subcloned into a baculovirus vector and expressed in the SF9 cell culture system. The expressed protein is the appropriate molecular mass for an intact βB2-crystallin and forms dimers appropriately. Modifications, including deletion of the amino and carboxy terminal arms, are being engineered into the βB2-crystallin protein; the effects of these modifications on its formation of homodimers and heterodimers with the normal and modified βA3-crystallin protein will be examined.

(5) Studies of phase transition properties of the γ-crystallin gene family have begun in collaboration with Dr. George Benedek, from the Massachusetts Institute of Technology, Boston. The bovine γB-crystallin has been modified at two of the four residues proposed to be critical for phase transition behavior. Phase transition analysis of the expressed, unmodified γ2-crystallin has begun in Boston.

(6) Ophthalmological diseases have been studied in humans by linkage analysis of restriction fragment length polymorphism markers. Diseases that we have mapped within the past year include Long QT syndrome, two types of autosomal dominant congenital cataracts, and Usher's syndrome type I. In addition, family collection has begun on Bieti syndrome in anticipation of carrying out linkage analysis. Clinical and genetic heterogeneity of Usher syndrome within the Acadian population has been explored in detail. Genetic analysis confirms the clinical impression that both type I and II Usher syndrome are found in the Acadian population and even within a single extended pedigree. The heterogeneity analysis described above implies that this must be due to segregation of two different and unlinked genes within this population.

Two genes causing Usher syndrome type I have been mapped. In the Acadian population of Louisiana, the genetic locus is on chromosome 11p, although in our British families, the gene is on chromosome 11q. These findings have been subjected to heterogeneity analysis using both the HOMOG2 program and the M-test and are significant at $p < .01$ under the most stringent analyses. This surprising finding implies that multiple genes can cause the rather specific clinical findings present in Usher syndrome. The location of the Usher syndrome gene on chromosome 11p has been studied in detail using fine linkage mapping and haplotype analysis. The Usher syndrome gene has been localized to a 5 cM interval between the markers D11S861 and D11S899. A YAC contig is being established over this region, and four YAC clones extending from D11S861 to

D11S902 form the minimal tiling path over the assembled region, with about 12 additional YAC clones incorporated into this contig.

(7) Several large families with autosomal dominant and recessive cataracts have been ascertained and studied clinically and samples collected. Genotyping of microsatellite markers has begun in four of these families with attention initially concentrated in regions around candidate genes. Two families have yielded significant lod scores, one on chromosome 2 and one on chromosome 17.

(8) The possible association of various genetic mutations and polymorphisms with visual function is being carried out. The effect of various polymorphisms in the red pigment genes with visual function in heterozygous females is being studied in collaboration with Dr. Raphael Caruso (Ophthalmic Genetics and Clinical Services Branch), and the effect of deletion of the glutathione S-transferase M1 gene on the frequency of cataracts is being investigated in collaboration with Dr. G. Maraini, from the Universita Degli Studi di Parma.

Significance to Biomedical Research and the Program of the Institute

Elucidation of the genetic defects causing visual disability will have implications far beyond the patient population suffering from the specific syndrome under study. Inherited diseases provide a means by which the molecular physiology and pathophysiology of the visual system may be understood, and this knowledge can then be applied to a broad spectrum of diseases as well as to the normal eye. This rationale also applies to the study of inherited diseases of which visual defects are only a small part. Thus, although our studies of myotonic dystrophy have already resulted in improved diagnostic abilities, the mechanism by which cataracts occur in this disease will provide insight into cataractogenesis in other hereditary syndromes as well as age-related and nonspecific cataracts.

Proposed Course

(1) Studies will continue on the structure-function relationships of lens crystallins, concentrating on the effects that modifications of the terminal arms, interconnecting peptide between the two domains, and surface charge and hydrophobic contact sites have on aggregation of both acidic and basic β-crystallins. We will also continue to explore the effects that modification of the Greek key motifs have on crystallin stability and, where applicable, lens transparency. In addition, the effects that modifications of γ-crystallin sequences have on the protein-phase transitions and their relationship to cold cataract will continue to be explored.

(2) Sample collection and linkage analysis of a variety of human diseases will continue. The main emphasis will be on inherited visual diseases, especially Usher syndrome type I and cataracts. Fine mapping and physical mapping of the Usher syndrome type IC gene on Chromosome 11p is being pursued, and candidate gene analysis will be initiated as soon as it would be meaningful. We are initiating fine mapping and candidate gene analysis of the autosomal dominant cataracts that we have mapped in families ascertained in collaboration with Dr. Muriel Kaiser-Kupfer and Dr. G.N. Rao and linkage analysis of the of autosomal recessive cataracts ascertained in collaboration with Dr. J.S. Murty in India. This will be coordinated with a new project categorizing and mapping expressed sequences of the human lens and the ongoing mechanistic studies on lens crystallins described above. Together, these projects should provide a coordinated effort to elucidate the mechanisms of cataractogenesis in the human lens.

(3) The studies of possible associations between mutations and polymorphisms of genes functioning in the vision process and visual function will continue. Sample collections from both Italy and the National Institutes of Health (NIH) will continue for the cataract epidemiological study. Patients will be re-

cruited for these studies through the NIH eye clinic.

(4) Families will be studied, and samples will be collected for a collaborative study of open angle glaucoma in Barbados. This will be carried out in collaboration with Dr. Christina Leske, from the State University of New York at Stony Brook, and will build on ongoing epidemiological studies.

NEI Research Program

Lens and Cataract—Molecular Genetics

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00149-21 LMOD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ultrastructure and Function of the Cells and Tissues of the Eye

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. Gerald Robison, Jr.	Ph.D.	Head, Section on Pathophysiology	LMOD, NEI
Others:	Nora Laver	M.D.	Special Volunteer	LMOD, NEI
	Jorge Jacot	Ph.D.	IRTA Fellow	LMOD, NEI
	Anne Groome	B.S.	Histology Technician	LMOD, NEI
	Joe Hackett	B.S.	Biologist	LMOD, NEI
	Evita Bynum	B.S.	Microbiologist	LMOD, NEI
	Joel Glover	B.S.	Biologist	LMOD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Pathophysiology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.0	2.0	4.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Diabetic retinopathy is the major cause of blindness in adults (20 to 74 years old) in the industrialized countries. Whereas systemic diabetes mellitus results from lowered availability and/or cellular recognition of insulin, the complications of diabetes, such as diabetic retinopathy, are caused by the chronic hyperglycemia itself. Exaggerating the hyperglycemic effect by feeding rats a galactose diet, we produced the first rat model for diabetic retinopathy. Intervention studies designed to simulate clinical trials were used to test the possibility of delaying, halting, or reversing retinopathy soon after the earliest capillary lesions could be documented. Weanling male Sprague-Dawley rats were divided into 10 groups, 4 of which received either normal lab chow or a 50 percent galactose diet with or without one of two aldose reductase inhibitors (ARIs: AL-3152 or WAY-121,509), and other groups that received 50 percent galactose for four, six, or eight months and then intervention either by addition of inhibitor or removal of galactose. From rats killed at four, six, eight, 16, 18, and 24 months, one retina was prepared for obtaining electron micrographs of capillary transections; the other was used for whole mounts of isolated retinal vessels. Images of whole and transected capillaries were captured and analyzed using computer hardware and programs specially designed for 1024-x 1024-x 8-bit resolution. Based on several quantitative assessments, including basement membrane thickness, PAS stain intensity, acellularity, dilation, tortuosity, length, and microaneurysms, the retinopathy was graded on a scale of 0 to 10. At 6 months, when intervention was begun, the untreated galactose-fed rats exhibited a 30 percent, statistically significant (p<0.01) increase in capillary basement membrane thickness and grade 1 retinopathy overall. By 18 months, the same group had grade 7 retinopathy, whereas the rats receiving intervention with an ARI-enriched or galactose-free diet exhibited only grade 2 retinopathy, and the rats fed control diet or galactose plus AL-3152 throughout the 18 months showed no retinopathy. At 24 months, the untreated rats exhibited grade 10 retinopathy, and both intervention groups had a grade 8.5 retinopathy. In conclusion, intervention at 6 months delays but does not halt or reverse the progression of galactose-induced retinopathy. We plan to attempt, by dietary manipulation, to produce rat models that develop the diabetic-like retinal angiopathies sooner. Also, using cell and organ culture, we will investigate the possible mechanisms.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The objective of this project is to use special diets *in vivo* and controlled media in cell cultures of ocular tissues to mimic the diabetic state to determine if diabetic-like tissue changes can be prevented by inhibitors of aldose reductase (AR).

Methods

Weanling male Sprague-Dawley rats were divided into groups, some of which received either normal laboratory chow or a 50 percent galactose diet with or without an AR inhibitor (AL-3152 or WAY-121,509 at 11 mg/kg/day), and other groups that received 50 percent galactose for four, six, or eight months and then intervention either by addition of inhibitor or removal of galactose. Rats were killed at four, six, eight, 16, 18, and 24 months. A new enzyme digestion procedure (elastase method), which was developed in this laboratory, was used on the retina of one of the eyes of each rat to remove all the retinal tissues except the vessels.

This provided a whole mount of the retinal vasculature and thus permitted the recognition of degenerated pericytes ("ghosts") and all the more advanced angiopathies by light microscopy. The retina of the other eye was sectioned and examined by electron microscopy. Images of whole and transected capillaries were captured and analyzed using computer hardware and programs specially designed for 1024 x 1024 x 8 bit resolution. Based on several quantitative assessments, including basement membrane thickness, periodic acid Schiff stain intensity, acellularity, dilation, tortuosity, length, and microaneurysms, the retinopathy was graded on a scale of one to 10. Tissue cultures of human, bovine, and canine retinal capillary pericytes and lens epithelial cells were used to investigate the mechanism of underlying diabetic angiopathies.

Major Findings

Vascular whole mounts prepared by our new enzyme digestion procedure exhibited multiple retinal angiopathies identical to those typical of human background diabetic retinopathy in the capillaries of rats fed galactose for 24 months. These did not occur in the retinas of rats fed a galactose diet with an AR inhibitor. AR was shown to be present in cultured retinal pericytes: (1) by immunohistochemistry using antibody against human placental AR, (2) by its activity using measurements of xylitol production in cells grown in a medium supplemented with xylose, and (3) by the detection of messenger ribonucleic acid for AR. There was a compromised proliferation rate in pericytes compared with endothelial cells when incubated in high (30 mM) sugar concentrations, suggesting toxicity of polyol at the cellular level. AR appears to be involved in all the retinal complications of diabetes, from pericyte degeneration to microaneurysms.

Significance to Biomedical Research and the Program of the Institute

Diabetic retinopathy is mainly a disease of retinal capillaries. Recently, potentially beneficial treatments and animal models have become available. However, demonstration of the earliest vessel lesions has relied on the 30-year-old trypsin digestion method for the isolation of retinal vessels. Until now, basic experimental studies and drug testing on diabetic retinopathy have been limited by the lack of reliable and convenient animal models. Now, besides the alloxan diabetic dog and the galactosemic dog, there is a galactosemic rat model. All this has been possible because AR is involved in diabetic retinopathy. AR, which has been implicated in sugar cataracts, certain corneal healing defects, and peripheral neuropathy of diabetic and galactosemic animals, now appears to be involved in all lesions of background diabetic retinopathy. Although

the normal physiological role of this enzyme in most tissues remains unknown, under the conditions of high plasma sugar concentrations encountered in diabetes and galactosemia, AR converts these sugars to their respective sugar alcohols (polyols). These polyols are not readily metabolized nor do they penetrate cell membranes easily. Thus, once formed at significant rates, they may accumulate to very high levels in cells, leading to hypertonicity, alteration of ion permeability, and eventual cell death with consequent tissue changes such as cataract formation. Treatment of diabetic or galactosemic rats with potent AR inhibitors such as sorbinil or tolrestat decreases the accumulation of polyols, which in turn appears to prevent the formation of cataracts in lenses, defective healing in scraped corneas, thickening of basement membranes in retinal capillaries, and decreased conduction velocity in nerves.

We have shown for the first time that the rat can be a good model for human diabetic retinopathy and that demonstration of early lesions can be improved by using a novel vessel preparation method. Pericyte loss, endothelial cell proliferation, microaneurysms, shunts, occlusions, dilations, and all the other microangiopathies that we found in the galactose-fed rat are identical to the histopathologies that characterize human background diabetic retinopathy. Until now, the only other experimental animal model has been the diabetic or galactosemic dog. We have shown for the first time that diabetic-like retinopathy in galactosemic rats can be prevented with an AR inhibitor.

Proposed Course

The following studies are proposed for fiscal year 1995. We will manipulate the rat diets to shorten the time when diabetic-like retinal angiopathies appear, thus improving the rat as a model for diabetic retinopathy. As needed, we will extend the intervention studies to determine how late one can interrupt the disease process and still obtain beneficial results by treatment with various AR inhibitors. Also, we will examine the early formation of intracellular vacuoles, cell transport systems, the mechanism of basement membrane synthesis, and the relations of these changes to AR in isolated retinal cells grown under diabetic conditions.

NEI Research Program

Retinal Diseases—Diabetic Retinopathy, Sickle Cell Retinopathy, and Other Vascular Abnormalities

Publications

Hallfrisch J, Xue Q, Michaelis OEIV, Robison WG Jr: Carbohydrate copper interactions in the development of cataracts [abstract]. FASEB meeting, Anaheim, California, April 24-28, 1994, vol. 8, issue 5, p A945.

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Laboratory of Molecular and Developmental Biology

Report of the Chief
Laboratory of Molecular and Developmental Biology

Joram Piatigorsky, Ph.D.

In its 13th year, the Laboratory of Molecular and Developmental Biology (LMDB) has continued to explore the molecular basis and consequences of gene expression in the eye and especially the lens. As always, our work strives to understand the eye from a molecular and cellular perspective while using this extraordinary organ as a model for general processes of evolution and development. We have also continued to exploit our findings that the lens crystallins are expressed outside of the eye, where they perform nonrefractive functions, to link gene expression in the eye with that in other parts of the body and with noneye and systemic diseases. The LMDB now comprises five sections, and their major accomplishments this year are detailed below. In addition to performing basic research, which is the primary function of each of the groups, the Transgenic Animal and Genome Manipulation Section, established last year, also produces transgenic mice as a service for the rest of the National Eye Institute (NEI). The development of this service has been a great success story, as can be gleaned from its report below.

Section on Molecular Genetics

This section, headed by Dr. Joram Piatigorsky, has continued to investigate the molecular basis of crystallin gene expression in the lens and other tissues. An important new development this year was the discovery that α-crystallins can be autophosphyrylated *in vitro*. This links these ubiquitous crystallins with the enzyme-crystallins and raises the possibility that they are involved in signal transduction pathways. Thus, the α-crystallins, which are molecular chaperons, can now be considered in a metabolic as well as a structural role. This provides new avenues of approach for investigating the functions of α-crystallin in the lens as well in nonlens and diseased tissues, for which there is a large and growing literature. A great deal of progress has been made on identifying the *cis*- and *trans*-regulatory controls for crystallin gene expression. Shared and tissue-specific *cis*-elements use have been identified for numerous crystallin genes. "Regulatory tissues prints," defined as the patterns of *cis*-elements used for expression of genes in different tissues, have been determined for the mouse αB-crystallin gene in lens, skeletal muscle, heart, and lung by performing experiments in cultured cells. Apart from its intrinsic interest, this information may be useful for designing promoters for gene therapy in the future. A particularly exciting development has been the finding that Pax-6, a paired-box homeodomain transcription factor, can act as a lens-specific activator for the chicken and mouse αA- and chicken δ1-crystallin genes. Because numerous eye mutations in species ranging from *Drosophila,* which have compound eyes, to humans, who have complex eyes, are due to lesions in Pax-6 or its homologue, it seems possible that the diverse crystallins may be linked by one or more common transcription factors. Pax-6 will clearly be investigated thoroughly next year. Many more findings on the molecular events of crystallin gene regulation have been made that will also be followed up, and these can be found in the annual reports form this section.

Finally, the presence of Dr. Joseph Horwitz, who has been on sabbatical leave from Jules Stein Eye Institute, UCLA School of Medicine, since January 1994, has led to an increased attention to biochemistry. One important development from this has been the finding of βB2-crystallin in nonlens tissues (retina, testis, brain). This was complemented by the finding that βB2-crystallin, like α-crystallin, can autophosphorylate *in vitro*. These data suggest strongly that the β-crystallins, and possibly their γ-crystallin relatives, have metabolic nonlens functions in addition to their refractive role in the lens. The second important development in protein biochemistry has been the discovery that the ratio of the two duck δ crystallin/argininosuccinate lyase polypeptides in the tetrameric native protein affects enzyme activity. Kinetic studies indicated a cooperative interaction between the δ1 and δ2 polypeptides. This provides a regulatory role for the inactive δ1 polypeptide and explains why it continues to be synthesized outside of the lens.

Transgenic Animal and Genome Manipulation Section

This section, headed by Dr. Eric Wawrousek, has continued to provide the NEI investigators with transgenic mice in support of their research efforts. This year, 155 new transgenic founder mice were generated from 34 deoxyribonucleic acid (DNA) constructs. More than 400 matings of transgenic mice were set up, and more than 4,000 mice were weaned, tagged, tail biopsied, and their DNA isolated and analyzed. This year also marked the successful launching of the group's embryo cryopreservation and banking program, providing the NEI with a reliable mechanism for long-term storage of valuable mouse lines. So far, 1,326 embryos from five transgenic lines have been frozen.

This section has also been conducting independent research in two areas, generating transgenic mouse models of ocular disease and probing the function of lens crystallins by deleting these proteins in "gene knockout" mice. In the first area, the section has created a transgenic mouse model of progressive ocular inflammation by expressing a modified form of human interleukin-1β in the eye under transcriptional control of the mouse αA-crystallin promoter. The animals are healthy, reproductively capable, and exhibit a consistent and reproducible pattern of ocular disease. In neonates, the eye is relatively normal, but inflammation with accompanying neovascularization of all eye tissues becomes evident and progresses to phthisis bulbi in adult animals. This will be a useful model for investigating many of the processes involved in chronic ocular inflammatory disease such as cytokine levels, receptor levels, arachidonic acid metabolite levels, cellular adhesion molecule expression, and systemic effects of the localized inflammation.

Significant progress has been made toward functionally deleting the α-crystallin gene family. Knockout vectors have been generated for mouse αA- and αB-crystallin and mouse αACRYPB-1, and approximately 20 embryonic stem cell clones have been isolated in which one allele of the αA-crystallin gene has been disrupted. Toward generating knockout mice, the group has mastered the skills for inserting ES cells into mouse embryos to generate chimeric mice. Many chimeric mice have been produced from unmodified ES cells, and it is expected that chimeric mice containing the αA-crystallin knockout ES cells will be produced very soon. The knockout mice will provide a unique resource for studying the function of the α-crystallins in lens and eye development and shed some light on their function in nonlenticular tissues. Development of the technology may one day also be a valuable resource for other researchers in the NEI.

Section on Regulation of Gene Expression

This section, headed by Dr. Ana Chepelinsky, has continued to study the expression of genes

encoding lens fiber membrane channels, which are of utmost importance for maintaining lens transparency. They have mapped several *cis* regulatory elements in the 5′ flanking sequence of the human major intrinsic protein (MIP) gene. Negative regulatory elements were found within the sequence -2840/-254, and positive regulatory elements were revealed between the sequence -70/-40 of the MIP gene.

A successful collaboration between the LMDB and the Laboratory of Immunology at the NEI allowed the development of transgenic mice and rats with constitutive expression of interferon gamma (IFN-γ) and major histocompatibility complex (MHC) class II in their eyes. These animals provide a comprehensive transgenic model system for elucidating the linkage between aberrant MHC class II expression and predisposition to autoimmunity, the role of IFN-γ in the treatment of inflammatory eye diseases, and cytokine signaling during embryonic eye development.

Section on Molecular Structure and Function

This section headed by Dr. Graeme Wistow, has continued to investigate the variety of proteins that can serve as functional crystallins. Work on ζ-crystallin has revealed a natural example of a consensus paired-domain binding site for Pax-6. This site is essential for lens-specific promoter function. Other projects have led to discoveries with significance beyond the lens. μ-Crystallin, originally discovered in marsupial lens, is expressed abundantly in retinal photoreceptors of humans and rodents, probably as an enzyme of glutamate or ornithine metabolism. Migration inhibitory factor (MIF), which was isolated as a developmental marker in chick lens, has been shown to have a general role in cell proliferation. It interacts with the retinoblastoma protein and is essential for progression through the cell cycle. Antisense suppression of MIF halts cell growth even in transformed mouse cells.

Section on Cellular Differentiation

This section, headed by Dr. Peggy Zelenka, has made progress in several areas in the past year. Several steps have been taken to follow-up the surprising discovery that postmitotic, differentiating lens fiber cells contain cyclin B, an activator of the cyclin-dependent kinase, $p34^{cdc2}$, that is normally associated with mitosis. Immunocytochemistry has shown that cyclin B is concentrated in the fiber cell nuclei as early as developmental day E6, and biochemical analysis indicates that cyclin B and $p34^{cdc2}$ complex may play a role in lens fiber cell denucleation, transgenic mice that carry the *wee1* gene under control of the γ-crystallin promoter. The product of the *wee1* gene is a kinase that inactivates $p34^{cdc2}$. Other studies carried out during this year have probed the similarities and differences between lens fiber cell differentiation and apoptosis. These have shown that cyclin B may be induced during apoptosis in PC12 cells as well as during lens fiber cell differentiation. Moreover, in apoptotic cells, cyclin B is complexed with a novel partner that is immunologically related to PCTAIRE-1, a member of the cyclin-dependent kinase family whose function is unknown.

Another similarity between lens fiber differentiation and apoptosis that was discovered is that the proto-oncogene, *c-jun* is activated during both processes. However, in differentiating cells, *c-jun* is downregulated after a few hours, while in apoptotic cells, it remains elevated until the cells die. Using a retrovirus vector to introduce *c-jun* into cultured cells, it was shown that elevated levels of *c-jun* promote cell death in the absence of growth factors. These studies open new opportunities for investigating the relationships between cell division and terminal lens fiber cell differentiation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00238-09 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Proto-oncogene Expression During Lens Differentiation and Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

PI:	Peggy S. Zelenka	Ph.D.	Head, Section on Cellular Differentiation	LMDB, NEI
Others:	Chun Yun Gao	M.D., Ph.D.	IRTA Fellow	LMDB, NEI
	Emmanuel Vacchiano	Ph.D.	IRTA Fellow	LMDB, NEI
	Anuradha Rampalli	Ph.D.	IRTA Fellow	LMDB, NEI
	Jaspreet Arora	Ph.D.	Visiting Fellow	LMDB, NEI
	Vijay Chauthaiwale	Ph.D.	Visiting Fellow	LMDB, NEI
	Graeme Wistow	Ph.D.	Head, Section on Molecular Structure and Function	LMDB, NEI

COOPERATING UNITS *(if any)*

Department of Surgery and Department of Anatomy and Cell Biology, New Jersey Medical and Dental College (Thomas Lysz, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Cellular Differentiation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.6	5.6	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project investigates the expression of proto-oncogenes and other cell cycle regulatory genes in the embryonic chicken lens to determine their relationship to cell growth, quiescence, and differentiation. The normal developmental profiles of six nuclear proto-oncogene messenger ribonucleic acid (mRNAs) (*c-myc*, *N-myc*, *c-fos*, *c-jun*, Rb, and p53) and the cell cycle regulatory protein, cyclin B, have been completed. The unexpected finding that cyclin B is present in postmitotic lens fiber cells and that it is complexed with $p34^{cdc2}$ suggests that lens fiber cell differentiation may involve aberrant progression through the cell cycle. To test this hypothesis, a line of transgenic mice has been produced that overexpresses Wee1 (a protein kinase that inhibits cyclin B/$p34^{cdc2}$) under control of the γ-crystallin promoter to target expression to postmitotic lens fiber cells. Preliminary analysis of lenses from this transgenic line indicates that lens fiber denucleation may be abnormal. Similarities between apoptosis and lens fiber differentiation prompted us to compare proto-oncogene expression in differentiating and apoptotic cells. The earliest difference we have detected between these two processes is deregulation of c-fos, c-jun, and c-myc expression during apoptosis. We have demonstrated that expression of these proto-oncogenes is strictly regulated by 12(S)HETE synthesis and are exploring the mechanism of this effect through analysis of the c-fos promoter. Candidate genes that may be regulated by these proto-oncogenes are being explored through transfection of cultured cells and deoxyribonucleic acid (DNA) binding studies. We have demonstrated that cyclin B is expressed during apoptosis of postmitotic PC12 cells, where it complexes with $p34^{cdc2}$ and with a novel, 40K member of the PCTAIRE family. The identity and function of this 40K protein are being investigated.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Vu Bui		IRTA Summer Fellow, LMDB, NEI
Graeme Wistow	Ph.D.	Head, Section on Molecular Structure LMDB, NEI and Function

Objectives

This project seeks to determine whether the expression of specific proto-oncogenes is altered during lens cell differentiation, and, if so, to determine the mechanism of gene regulation and the function of the corresponding proto-oncogene products in the developing lens. The objective is a greater understanding of the mechanisms underlying lens cell growth and differentiation.

Methods

Techniques of molecular biology are used in conjunction with traditional techniques of cell biology. Conventional methods are used for analysis of proteins and nucleic acids, including polyacrylamide gel electrophoresis, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) isolation, polymerase chain reaction (PCR), nucleic acid hybridization, *in vitro* transfection, *in situ* hybridization, immunocytochemistry, and immunoblotting. DNA/protein interactions are studied using DNase I footprinting, electrophoretic mobility shift assays, and ultraviolet crosslinking.

Studies use lens epithelia and lens fibers of embryonic chickens, explants of embryonic chicken lens epithelia, primary cultures of embryonic chicken lens epithelial cells, and other avian and mammalian cell lines. In addition, transgenic mice are produced to test the function of proto-oncogenes and cell cycle regulatory proteins in the lens *in vivo.*

Major Findings

Terminal differentiation of lens fiber cells is marked by chromatin condensation, abrupt dissolution of the nuclear lamina, vesicularization of the nuclear membrane, and complete degradation of the nucleus and other organelles. Because these events resemble the chromosomal condensation and nuclear envelope breakdown associated with mitosis, we have investigated whether a similar biochemical mechanism may be involved by testing for the expression and activity of cyclin B/p34^{cdc2} complexes in differentiating lens fiber cells. A coupled reverse transcription/PCR (RT/PCR) using RNA from embryonic chicken lens fibers amplified a product of the expected size, which was identified as cyclin B2 by sequencing. *In situ* hybridization localized cyclin B messenger RNA (mRNA) to the superficial nucleated fiber cells, and immunocytochemistry with antichicken cyclin B2 antiserum showed positive staining in lens fiber cell nuclei. Immunoblotting of lens fiber proteins purified by affinity chromatography on p13-agarose beads identified both p34^{cdc2} and cyclin B, indicating that these proteins are present as a complex.

Moreover, fiber cell proteins purified on p13-agarose beads showed histone H1 kinase activity, which was enhanced by phosphatase digestion. These results demonstrate that active cyclinB/p34^{cdc2} complexes are present in differentiating lens fiber cells before denucleation and support the possibility that phosphorylation of specific nuclear substrates by p34^{cdc2} may be responsible for the denucleation of lens fiber cells during terminal differentiation.

Although denucleation of lens fiber cell differentiation resembles mitosis, other features such as degradation of DNA to oligonucleosomes resemble apoptosis. This similarity and our discovery of cyclin B in differentiating lens fibers led us to explore the involvement of cyclin B in apoptosis. These studies were performed using neuronally differentiated PC12 cells forced to undergo apoptosis by withdrawal of nerve growth factor. Cyclin B

mRNA increased approximately 10-fold four days after NGF withdrawal, as indicated by competitive RT/PCR. Cyclin B protein also increased during this period, as indicated by immunoblotting. Immunoprecipitation with anticyclin B antibody demonstrated that cyclin B was associated with H1K activity, which reached a maximum five days after NGF withdrawal. When proteins immunoprecipitated with anticyclin B antibody were immunoblotted with anti-PCTAIRE antibody, proteins with apparent molecular weights of 34kDa and 40kDa were detected. The 34kDa protein was identified as $p34^{cdc2}$ on the basis of immunoreactivity with antibody against the C-terminal portion of $p34^{cdc2}$. The 40kDa protein reacted strongly with an antibody against the C-terminal region of PCTAIRE-1 and failed to bind to p13-agarose beads, suggesting that it is a member of the PCTAIRE family. Kinase assays of anticyclin B immunoprecipitates from apoptotic cell lysates that were first immunodepleted of $p34^{cdc2}$ indicated that significant H1K activity was associated with the cyclin B/40kDa protein complex. These findings suggest that certain members of the PCTAIRE family may function as cyclin-dependent kinases in apoptotic cells. We are currently investigating expression of the 40kDa protein in differentiating lens fibers and in apoptotic retinal cells.

The relationship between lens differentiation and apoptosis has been further probed by Dr. Anu Rampalli, who has compared expression of several proto-oncogenes and cell cycle markers in embryonic chicken lens epithelial explants during differentiation or apoptosis *in vitro*. Interestingly, proto-oncogenes *c-fos*, *c-jun*, *c-myc*, and *p53* are sequentially upregulated during both processes. However, in differentiating cells, these increases in mRNA expression are transient, although in apoptotic cells they are not. Loss of regulation of *c-fos* and *c-jun* mRNA expression, which occurs within five hours in culture, is the earliest difference detected between differentiating and apoptotic cells. Moreover, Dr. Emanuel Vacchiano has demonstrated that overexpression of *c-jun* in chicken lens epithelial cells in the absence of growth factors greatly increases the rate of cell death due to apoptosis. Thus, deregulated expression of *c-jun* appears to be an early step in the pathway leading to apoptosis of lens epithelial cells.

Two experimental approaches have been used to explore the functional role of proto-oncogenes such as *c-fos*, *c-jun*, and *c-myc* in the lens. Dr. Vijay Chauthaiwale has used a candidate gene approach to determine whether *c-myc* is involved in regulation of the α-enolase/τ-crystallin gene. The promoter of this gene contains a potential *c-myc* binding site that is conserved in both duck and human. Dr. Chauthaiwale has demonstrated that *c-myc* binds to this site by immunoblotting with anti-(*c-myc*) antibody following electrophoretic mobility shift assay of lens nuclear proteins bound to an oligonucleotide containing the potential binding site. Immunoreactive bands were detected by enhanced chemiluminescence. Interestingly, a mutated oligonucleotide containing substitutions at the two central nucleotides of the potential binding site showed unimpaired binding of *c-myc* but greatly diminished binding of a faster migrating band. Transfection studies using a α-enolase/τ-crystallin promoter construct bearing this same mutation had previously shown that this mutation increased basal transcription of the promoter and abolished inducibility by *c-myc*. Together these results suggest that *c-myc* competes with a negative regulatory factor for occupation of the same site. Dr. Chauthaiwale has now demonstrated that the fast migrating band that is diminished by the mutation comigrates with a band produced by *in vitro* translated max proteins, suggesting that max/max homodimers may be the negative regulatory agent. This possibility is being explored by means of cotransfection studies with *c-myc* and *c-max* plasmids.

The other experimental approach used to study the targets of proto-oncogenes in the lens is overexpression of these genes and negative dominant mutations using retroviral vectors. We have used the avian retroviral vector RCAS to overexpress wild-type chicken *c-jun*, or a deletion mutant of chicken *c-jun* (*jun*Δ7) lacking the DNA binding region, to

investigate the possible role of *c-jun* in lens epithelial cell proliferation and differentiation. Both constructs were efficiently expressed in primary cultures of embryonic chicken lens epithelial cells. Overexpression of *c-jun* increased the rate of cell proliferation and greatly delayed the appearance of "lentoid bodies," structures that contain differentiated cells expressing fiber cell markers. Excess *c-jun* expression also significantly decreased the level of $\beta_{A3/A1}$-crystallin mRNA without affecting αA-crystallin mRNA. In contrast, the mutated protein *jun*Δ*7* had no effect on proliferation or differentiation but markedly increased the level of αA-crystallin mRNA in proliferating cell cultures. These results suggest that *c-jun* or *jun*-related proteins may be negative regulators of αA- and βA3/A1-crystallin genes in proliferating lens cells.

Studies of the role of 12-lipoxygenase in regulating lens epithelial cell proliferation have made significant advances in the past year. To determine whether products of this pathway are involved in lens cell proliferation, we measured the effect of 12-lipoxygenase inhibitors on endogenous 12-hydroxyeicosatetraenoic acid (HETE) production and epidermal growth factor (EGF)/insulin-stimulated DNA synthesis and proto-oncogene expression in cultured neonatal rat lens epithelial cells. Incubation of neonatal rat lenses in EGF plus insulin, which stimulated endogenous 12-HETE production eightfold to 10-fold, also produced a transient induction of *c-fos* and *c-myc* mRNAs after two to three hours, followed by a round of DNA synthesis approximately 20 hours later. The lipoxygenase inhibitor cinnamyl 3,4 dihydroxy-α-cyanocinnamate (CDC) strongly inhibited both the endogenous 12-HETE synthesis and growth-factor stimulated DNA synthesis with half-maximal inhibition between 10-20 μM. CDC (10μM) also inhibited the expression of *c-fos* and *c-myc* mRNA and to a lesser extent, *c-jun* mRNA. The inhibitory effects of CDC on proto-oncogene expression and DNA synthesis were prevented by 0.3 μM 12-HETE but not by equivalent concentrations of either 5- or 15-HETE. These findings suggest that endogenously synthesized 12-HETE may mediate EGF/insulin-stimulated DNA synthesis in neonatal rat lens epithelial cells by regulating proto-oncogene expression.

If 12-HETE also regulates human lens epithelial cell proliferation, inhibition of 12-lipoxygenase activity may provide a means of preventing unwanted proliferation following cataract extraction. We have, therefore, examined expression of this enzyme in human lens epithelial cells. Presence of 12-lipoxygenase mRNA was demonstrated in epithelial cells of adult and neonatal human lenses by RT/PCR and sequencing of the PCR product. The presence of the corresponding protein was demonstrated in cultured neonatal human lens epithelial cells by immunoblotting with an antibody raised against human platelet 12-lipoxygenase. A medium derived from human lens epithelial cell cultures was shown to contain 12-HETE by radio immunoassay. Experiments are in progress to test whether inhibition of 12-lipoxygenase blocks DNA synthesis in cultured human lens epithelial cells as in the neonatal rat lens. Dr. Jaspreet Arora has also demonstrated the presence of the 12-lipoxygenase pathway in cultures of rabbit lens epithelial cells (N/N10003 cells). This system will be used to investigate the mechanism of the 12-HETE effect on proto-oncogene expression in the lens.

Proposed Course

The following studies are in progress or are proposed for fiscal year 1995:

(1) The hypothesis that the cyclinB/p34^{cdc2} kinase is responsible for nuclear loss in differentiating lens cells will be tested by analysis of several lines of transgenic mice that express the *wee1*$^{+}$ kinase in lens fiber cells. This kinase inactivates the cyclinB/p34^{cdc2} kinase and would be expected to delay or prevent nuclear loss if, indeed, cyclinB/p34^{cdc2} is required.

(2) The finding that cyclin B binds to a novel 40kDa PCTAIRE-like protein in apoptotic cells will be explored further. The identity of the protein will be examined by protein microsequencing, and an attempt will be made

to clone the corresponding complementary DNA. Expression of this protein will be examined in the lens during differentiation and in a variety of ocular cell types during apoptosis.

(3) The effect of *c-myc* on transcription of the T-crystallin/α-enolase gene will be examined further by cotransfection studies with *max* and *myc* plasmids.

(4) The collaborative effort with Dr. Thomas Lysz, from the University of Medicine and Dentistry of New Jersey, will be continued to determine whether the 12-lipoxygenase pathway plays a role in regulating DNA synthesis in human lens epithelial cells.

(5) The possible role of posttranslational modifications of Rb and p53 proteins in lens cell differentiation will be explored using differential extraction techniques and specific antibodies. The relationship between migration inhibitory factor and modified forms of Rb will be examined in differentiating cells.

NEI Research Program

Lens and Cataract—Molecular Genetics

Publications

Dash A, Chung S, and Zelenka PS: Expression of HSP70 mRNA in the embryonic chicken lens: association with differentiation. *Exp Eye Res* 58, 381-387, 1994.

Gao CY, Bassnett S, Zelenka PS: Cyclin B, $p34^{cdc2}$, and H1-kinase activity in terminally differentiating lens fiber cells. *Develop Biol*, in press.

Lysz TW, Arora JK, Lin C, Zelenka PS: 12(S)-Hydroxyeicosatetraenoic acid regulated DNA synthesis and protooncogene expression induced by epidermal growth factor and insulin in rat lens epithelium. *Cell Growth Diff*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00126-13 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Crystallin Genes: Structure, Organization, Expression, and Evolution

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Joram Piatigorsky	Ph.D.	Chief	LMDB, NEI
Others:	Sharmilla Basu	Ph.D.		
	James B. Brady	Ph.D.	IRTA	LMDB, NEI
	Sambath Chung	B.A.	Technician	LMDB, NEI
	Ales Cvekl	Ph.D.	Visiting Fellow	LMDB, NEI
	Melinda K. Duncan	Ph.D.	IRTA	LMDB, NEI
	Peter Frederikse	Ph.D.	Senior Staff Fellow	LMDB, NEI
	Rashmi Gopal-Srivastava	Ph.D.	Staff Fellow	LMDB, NEI
	John I. Haynes, II	Ph.D.	IRTA	LMDB, NEI
	John G. Ilagan	B.A.	Howard Hughes Medical Institute/ NIH Fellow	LMDB, NEI

(Additional personnel listed under Program Description.)

COOPERATING UNITS *(if any)*

Jules Stein Eye Institute, UCLA School of Medicine (J. Horwitz, Ph.D.); University of Waterloo (Jacob Sivak, Ph.D. and Judith West Mays, Ph.D.); University of Nijmegen (Wilfried de Jong, Ph.D.); National Institute of Diabetes and Digestive and Kidney Diseases, NIH (Emery H. Bresnick, Ph.D.); National Cancer Institute, NIH (John N. Brady, Ph.D. and Fatah Kashanchi, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Molecular Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
15.70	13.15	2.55

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structure, expression, and evolution of the crystallin genes of vertebrates and invertebrates are being studied. The following advances have been made. Pax-6 activates the chicken and mouse αA-crystallin promoter and the chicken δ1-crystallin enhancer. The chicken αA promoter contains a composite element that suppresses activity in fibroblasts by binding USF and AP-1 proteins (JunD and Fra2) and activates promoter activity in lens by binding USF and CREB\CREM proteins. USF also acts as a negative regulator of the chicken αA promoter by binding to another, downstream element. USF also binds to the δEF1 site of the chicken δ1-crystallin enhancer, where it probably contributes to the activation of this gene. CREB\CREM cooperates with αA-CRYBP1 and Pax-6 to activate the mouse αA promoter in lens cells. Binding studies suggest that HSF may also be involved in chicken αA and mouse γF promoter activity. Expression of the mouse αB-crystallin gene in different tissues utilizes both shared and tissue-specific control elements, the exact pattern being called its "regulatory tissue print." Elements for lens-specific expression have been narrowed to positions -101/+30 in the chicken βB1 gene and -143/+22 in the chicken βBA3/A1 gene in transgenic mouse experiments. αA, αB and βB2 were shown to be able to autophosphorylate, raising the possibility that they are involved in a signal transduction pathway. Close linkage was found for chicken βB1 and βA4, and evidence was obtained indicating that mammalian βB2 is expressed in nonlens cells. A CR1 element was found in the intergenic spacer of duck δ-crystallin; the duck δ1 and δ2 polypeptides were shown to interact cooperatively to modulate argininosuccinate lyase activity of the tetramer. The jellyfish J3-crystallin gene has been cloned and shown to contain at least six introns.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Joseph Horwitz	Ph.D.	Jules Stein Eye Institute
Cynthia J. Jaworski	Ph.D.	Staff Fellow, LMDB, NEI
Marc Kantorow	Ph.D.	Staff Fellow, LMDB, NEI
Xuan Li	Ph.D.	Visiting Associate, LMDB, NEI
Joan B. McDermott	Ph.D.	Biologist, LMDB, NEI
Barbara Norman	M.S.	Chemist, LMDB, NEI
Christina M. Sax	Ph.D.	Senior Staff Fellow, LMDB, NEI
Stanislav I. Tomarev	Ph.D.	Visiting Scientist, LMDB, NEI

Objectives

The objective of this project is to understand the structure, organization, expression, and evolution of the gene families encoding the lens crystallins in the animal kingdom. Particular attention is given to the regulation of crystallin gene expression in the developing lens and, in the case of multifunctional crystallins and enzyme-crystallins, in nonlens tissues.

Methods

Conventional methods used for analysis of proteins and nucleic acids include polycrylamide and agarose gel electrophoresis, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) isolation, molecular hybridization (Southern and Northern blots), complementary DNA (cDNA) and gene cloning, DNA sequencing, recombinant DNA (rDNA) construction and mutagenesis, *in situ* hybridization, expression of recombinant DNAs in transfected cells and transgenic mice, polymerase chain reactions (PCRs), primer extension and S1 protection experiments, *in vitro* and *in vivo* footprinting, gel mobility shift analysis, chromatographic purification of proteins, and Western immunoblotting.

Major Findings

α-Crystallins. -αA-CRYBP1 is a zinc-finger transcription factor that binds to the mouse αA-crystallin gene at positions -66/-57 of its 5′ flanking sequence. We have previously cloned a partial cDNA encoding this protein. This year it was shown that the αA-CRYBP1 protein exists in different sizes in lens and fibroblasts, suggesting the possibility that posttranscriptional mechanisms regulate its binding or functional activity in a tissue-specific fashion. Moreover, we have established the full-length sequence for this 9.5 kb messenger RNA (mRNA). The deduced protein contains two sets of consensus C_2H_2 zinc-fingers (one set at the N-terminal region and one set at the C-terminal region) as well as a nonconsensus zinc-finger motif in the center. The 70 kbp gene has also been cloned and its 10 exon structure established. αA-CRYBP1 cDNAs were isolated from adult mouse brain and testis as well as from cell lines derived from mouse lens and muscle. An alternatively spliced αA-CRYBP1 cDNA was demonstrated in the muscle cell line, and a unique, smaller cDNA was found in testis. An antisense expression construct derived from an αA-CRYBP1 cDNA was introduced into the αTN4-1 transformed mouse lens cell line, and this significantly inhibited expression from a transfected heterologous promoter that used the αA-CRYBP1 binding site. This provides the first direct evidence (apart from mutagenesis tests) that αA-CRYBP1 is a positive transcription factor contributing to expression of the αA-crystallin gene in the mouse.

Transgenic mice experiments have established that the DE-1 (-111\-106) and αA-CRYBP1 (-66\-57) regulatory elements of the mouse αA-CRYBP1 are functionally redundant. Elimination of either one alone does not affect the lens-specific activity of the promoter; however, elimination of both simultaneously eliminates promoter activity. Footprinting experiments have shown that these regulatory sites are occupied in lens cells that are expressing the αA-crystallin gene and in fibroblasts that are not expressing the gene. It is not yet known whether the proteins binding

to these regions are identical in the two cell types or whether cofactors are involved. Mutagenesis, cotransfections, and immunoshift experiments have provided strong evidence that DE-1 binds the cAMP responsive transcription factor CREB/CREM and is thus a functional CRE site. Consequently it has been called DE-1/CRE. These footprinting experiments have also revealed the binding of proteins to two other putative regulatory regions called PE1 (involving the TATA box and immediately downstream thereof; -35/-19) and PE2 (+24\+43). Mutagenesis, transfection, and protein-binding experiments have shown that PE1 and PE2 are indeed important regulatory regions for the expression of this gene. A particularly unexpected finding is that site-specific elimination of the TATA box of the mouse αA-crystallin promoter fused to the chloramphenicol acetyltransferase (CAT) gene did not prevent this transgene from being expressed specifically in the lens of transgenic mice. Finally, negative-acting and protein-binding elements have been identified within the -1556/-1165 sequence of the mouse αA-crystallin 5′ flanking sequence.

Protein binding, mutagenesis, cotransfection, and immunoshift experiments have indicated that Pax-6, a paired-box/homeodomain protein expressed highly in the lens in the early stages of development of the mouse and chicken, is an important transcription factor affecting the lens-specific expression of the mouse and chicken αA-crystallin gene. Pax-6 also appears to activate the chicken δ1-crystallin enhancer. A number of studies have implicated Pax-6 as a universal regulator of eye development in both vertebrates and invertebrates, including both complex and compound eyes. Our studies provide the first evidence for specific target genes activated by Pax-6.

The chicken αA-crystallin promoter\enhancer (-153\+77) has been divided into at least five functional regions (A-E) by protein binding, mutagenesis, immunoshift, and transfection experiments. This region has been shown to be composed of a complex array of positive and negative elements involving Pax-6, USF, CREB/CREM and AP1 proteins. Sites A (-148\-139, binds USF) and B (-138\-132, binds CREB\CREM in lens and Fra-2 and\or JunB in fibroblasts) comprise a composite element that activates transcription in lens and represses transcription in fibroblasts. Sites C (-128\-101) and E (-56/-41) bind Pax-6 only in lens, which appears to be of central importance for the lens-specific expression of this gene. Site D (-102\-93) binds USF and is a negative element.

Finally, electrophoretic mobility shift experiments using specific antibodies have provided evidence that heat shock transcription factors bind to 5′ flanking sequences that are involved in the regulated expression of the chicken αA-crystallin and mouse γF-crystallin genes in the lens. These regions include positions -157/-136 of the chicken αA gene and positions -53/-23 of the γF gene. Indirect evidence for the use of heat shock factors for crystallin gene expression has also been obtained in stably transformed and transfected K562 cells treated with hemin, which is known to induce HSF2, a heat shock transcription factor known to be expressed in the lens.

The αB-crystallin gene contrasts with the αA-crystallin gene in that, although lens-preferred, it is also expressed to a relatively high degree in numerous other tissues and is inducible by heat and other physiological stresses. In 1991, we identified a strong muscle and weak lens enhancer between positions -426 and -257 of the 5′ flanking region of the gene. Last year, we identified by site-specific mutagenesis experiments four regulatory elements (αBE-1, αBE-2, αBE-3m and muscle regulatory factor [MRF]) within this enhancer. The MRF site is an E-box and was shown by transfection and transgenic mouse experiments to be muscle specific and to be activated by MyoD, myogenin, or another member of this family of transcription factors.

This year, we showed by DNase I footprinting experiments that there is another regulatory element (αBE-4) located between αBE-1 and αBE-2 that is used specifically in the heart. A series of footprinting, mutagene-

sis, and transfection experiments have resulted in the following conclusions: αBE-1 and αBE-2 are used for expression in muscle, heart, lung, and lens; αBE-4 is heart-specific and binds a protein identical or related to the serum response factor; αBE-3 is used by muscle, lens, and heart; MRF is used by muscle and heart and maybe by lung. Recent evidence suggests that MRF may bind different proteins in the different tissues.

Last year, we reported on another sequence called LSR (for lens specific region), that is located downstream of the enhancer at position -147\-118 of the mouse αB-crystallin gene. This element binds nuclear proteins that appear specific to the lens and may account for the high expression of the αB-crystallin gene in the lens. Site-specific mutagenesis and transfection tests have supported the importance of this sequence for lens expression. Experiments with transgenic mice have shown that the -164\+44 sequence, which contains the LSR, fused to the CAT reporter gene is sufficient to direct lens-specific expression of CAT; by contrast, the -426\+44 sequence containing the enhancer directs CAT expression to the lens, skeletal muscle, heart, and, to a much smaller extent, brain and lung. Thus, the differential expression of the αB-crystallin gene is regulated by a combination of tissue-specific and shared *cis*-control elements. Moreover, the shared control elements do not necessarily bind the same nuclear proteins in the different tissues. We have called the combination of regulatory elements used for expression of the αB-crystallin gene in any specific tissue its "regulatory tissue print" and, apart from its intrinsic interest, has obvious importance for gene therapy.

We have discovered an unexpected autophosphorylation ability of the αA- and αB-crystallin polypeptides. This raises the possibility that these crystallins are involved in a signal transduction pathway that ultimately could affect expression of crystallin genes in the lens. We have also shown that the detergent deoxycholate generates dimers and tetramers of the αA-crystallin polypeptides and that these small aggregates have approximately 10 times more autophosphorylation ability than the larger molecular weight aggregates present without the detergent. Interestingly, the αB-crytallin polypeptides also form dimers and tetramers in the presence of deoxycholate, but their autophosphorylation ability is not increased, suggesting a fundamental difference between the structure and possibly function of the αA and αB-crystallin polypeptides. Recent collaborative studies with Dr. Wilfried de Jong, from the University of Nijmegen, The Netherlands, have shown that the autophosphorylation of αA does not occur on serine 122, the amino acid that is phosphorylated in the cAMP-dependent reaction occurring on this polypeptide. This is consistent with our recent finding that the chicken αA-crystallin polypeptide is also autophosphorylated although it has an alanine substituted for serine at position 122.

β-crystallins. Our previous transfection and footprinting experiments have identified numerous control elements in the -152\+30 sequence of the chicken βB1-crystallin gene. Transgenic mice have now been produced that have established that the -152\+30 sequence is sufficient for lens-specific expression. Further deletions have shown that even the -101\+30 sequence is sufficient for lens-specific expression of the transgene in transgenic mice. By contrast, the -52\+30 sequence is not capable of expressing the transgene. Transgenic mice carrying various mutations in the 152\+30-CAT transgenes are being produced to identify specific control elements.

Analysis of the 5′ flanking region of the cloned chicken βB1-crystallin genomic fragment revealed, unexpectedly, the presence of the βA4-crystallin gene. This gene is arranged head to head with the βB1-crystallin gene, with approximately 2 kbp of spacer DNA separating the two genes. The βA4 gene was sequenced and shown to contain, like the βB1 gene, six exons, with the first being noncoding. A βA4-crystallin cDNA has been isolated from the embryonic chicken lens, indicating that this gene is expressed. The close head-to-head linkage of the βB1 and βA4 crystallin genes raises interesting possibilities with

respect to the regulatory mechanisms used for their expression.

We have shown previously that transcriptional activating sequences lie upsteam of the chicken βA3/A1-crystallin gene between positions -382 and -143, which contains a complex transcriptional enhancer between positions -287 and -254. This year we have performed DNase I footprint analysis revealing extensive protein binding in this region. Full enhancer activity requires sequences upstream and downstream of -270, although sequence -270\-254 has some enhancer activity by itself. There is an AP-1 consensus binding site at position -264\-258. Multiple proteins in lens nuclear extracts, including members of the AP-1 and ATF\CREB families, bind oligonucleotide -271\-251 in gel shift assays. Finally, the -143\+22 sequence, which lacks the enhancer, fused to the CAT gene in transgenic mice is sufficient for lens-specific expression. Thus, the -287/-254 enhancer is not required to direct expression of the βA3/A1-crystallin gene in the lens.

During his sabbatical year in the LMDB, Dr. Joseph Horwitz, from the Jules Stein Eye Institute, UCLA, has been exploring the possibility that βB2-crystallin is expressed in nonlens tissues. He had obtained strong preliminary evidence that this was the case before starting his sabbatical here. This would be of great interest because there is no unequivocal evidence that any of the β/γ-crystallins have nonlens functions as do the α-crystallins or the enzyme-crystallins. At present, Western immunoblotting of retina, testes, and brain from rats support the idea that βB2-crystallin is present outside of the lens. Amino acid sequencing of the putative βB2 polypeptide from bovine retina provided strong support for the expression of this protein in that tissue. The only caveat that must be kept in mind is the possibility that the retinal βB2-crystallin was derived by contamination from the lens. PCR data support the existence of βB2 mRNA in testes and brain of the rat, and Northern blots are presently being performed. Initial experiments on purified bovine βB2-crystallin indicate that this polypeptide is capable of autophosphorylation like the α-crystallin polypeptides; this autophosphorylation reaction has not been characterized yet. Autophosphorylation ability would open the possibility that βB2-crystallin is involved in some type of metabolic or signal transduction pathway, providing an explanation for its nonlens expression as well as introducing new considerations for the role(s) of this polypeptide in the lens.

δ-crystallin/argininosuccinate lyase (ASL). There are two linked ASL/δ-crystallin genes in the chicken, with the 5′ gene being specialized for expression in the lens. The 5′ gene encodes a polypeptide that lacks ASL activity; the 3′ gene encodes a polypeptide that has ASL activity. A similar situation exists in the duck, with the exception that the two ASL/δ-crystallin genes are equally expressed in the lens in this species. The 5′ δ1 gene is expressed to a limited extent in nonlens tissues in both species. This year, cloning experiments have shown that the 4.6 kbp intergenic spacer of the δ-crystallin genes in the duck is 79 percent identical to the 4 kbp spacer in the chicken, except for the existence of a 615 bp CR1 element, highly reiterated in the duck genome. Further sequence analysis revealed that intron 3 of the duck ASL/δ2 gene is 80 percent identical to intron 3 of the chicken δ1 and ASL/δ2 genes; the identity rises to 93 percent in the region of the chicken δ1 enhancer core located within the third intron. These findings raise the speculation that the CR1 repetitive element in the duck intergenic spacer plays a role in the high expression of the ASL/δ2 gene in the lens in this species, perhaps by diminishing the effectiveness of a silencer element that remains active in the chicken.

Footprinting and transfection experiments have shown that the chicken δ1-crystallin enhancer has at least two functional Pax-6 binding sites. The finding that Pax-6 activates the δ1-crystallin enhancer shows for the first time that the α- and δ-crystallin genes may be expressed by similar mechanisms. This idea is further supported by another recent finding, namely that USF binds to the δEF1 site in the

δ1 enhancer. USF participates in the regulation of the chicken αA-crystallin gene. The δEF1 site binds the negative regulator, δEF1, in nonlens tissues to suppress gene activity. We envision that USF binding to the δEF1 site activates δ1 expression in the lens, and δEF1 binding to this site represses δ1 expression.

Native δ-crystallin is a tetrameric protein. In 1979, we showed by SDS-polyacrylamide gel electrophoresis that the five major isoelectric forms of duck native δ-crystallin result from differences in the relative amounts of the two major polypeptide bands (about 49kD and 50kD). Current protein sequencing experiments have established that the 49kD polypeptide is encoded by the δ1-crystallin gene and the 50kD polypeptide is encoded by the ASL/δ2-crystallin gene. Moreover, we have demonstrated that the ASL activity of the isolated isoelectric forms of duck δ-crystallin is directly related to the relative amount of δ2 polypeptide present in the native tetrameric protein. Our new data indicate that the two δ-crystallin polypeptides interact cooperatively to modulate ASL activity. This provides a dominant negative role to the δ1-crystallin polypeptide in the regulation of cellular ASL activity within tissues.

Interesting cDNAs. We have cloned a partial cDNA for prox1 from an embryonic chicken lens library. This homeodomain protein is known to be expressed at high concentrations in the developing mouse lens; its *Drosophila* homologue, prospero, is expressed in the lens secreting cone cells of the developing compound eye of this invertebrate. Thus, prox1/prospero is another candidate transcription factor for controlling the high expression of eye genes throughout the animal kingdom, as Pax-6. A genomic clone for prox1 has also been cloned from humans that is in the process of being characterized.

A zinc-finger cDNA has been cloned from a newborn mouse lens library. The mRNA for this gene is also present in numerous other tissues. It encodes a 555-amino acid protein that contains nine C-terminal zinc-fingers and an N-terminal domain found in a subset of C_2H_2 zinc-fingers known as the Kruppel-associated box (KRAB). The amino acid sequence located between the KRAB domain and the zinc-finger shows an unexpected similarity to human profilagrin, a protein expressed in differentiating epidermal cells. Sequences that hybridized to this cDNA are detectable in 10 other mammalian species.

Invertebrate crystallins. We are continuing to explore the structure and expression of the squid S-crystallin genes, which are descended from glutathione S-transferase. A collaborative study with Dr. Richard N. Armstrong, from the University of Maryland, has led to the preliminary x-ray structure of squid glutathione S-transferase. We have cloned this cDNA from the digestive gland and its respective gene of the squid last year. One of the interesting aspects of the squid glutathione S-transferase is that it is approximately 100 times more active than its mammalian homologues in *in vitro* experiments. The three-dimensional structure of squid glutathione S-transferase is relevant to lens in view of the family relationship between this metabolic enzyme and the S-crystallins that make up approximately 90 percent of the squid lens protein.

A collaborative immunolocalization study with Dr. Jacob Sivak, from the University of Waterloo, Canada, has demonstrated that during development S-crystallins accumulate first in the posterior lens primordium and subsequently in the anterior lens primordium and their respective lentigenic cells and connecting lentigenic processes. Examination of adult lens and lentigenic cells of the squid suggested that squid lens crystallins are synthesized in the mature squid.

Another major interest of this section is the crystallins of the cubomedusan jellyfish. These primitive metazoan organisms have well developed eyes called ocelli, which contain cellular lenses. We have previously reported that the cubomedusan lenses contain three crystallins (J1, J2, and J3) with molecular sizes near 35kDa, 20kDa, and 19kDa, respectively. Last year, we cloned the cDNAs and

genes for J1-crystallins, which make up a family of three very closely similar polypeptides, each encoded by a separate intronless gene. This year, we have cloned the cDNA and gene for J3-crystallin. In contrast to J1-crystallin, J3-crystallin appears to contain only a single gene that has at least six introns. Another new and exciting development is the cloning of a genomic fragment, obtained by PCR, for cubomedusan Pax-6.

Significance to Biomedical Research and the Program of the Institute

The crystallins comprise a diverse family of differentially expressed proteins that are required for the optical and functional properties of the transparent lens. Understanding the structure, function, and evolution of these protein families and their genes contributes to our knowledge of embryonic development, eukaryotic gene expression, cell differentiation, molecular evolution, cataract, and the visual system. That crystallins are multifunctional proteins expressed in lens and nonlens tissues adds another dimension of interest and has implications for metabolism, cell biology, and drug and gene therapy.

Proposed Course

The following studies are proposed for fiscal year 1995.

(1) Continue studying *cis*-acting elements and *trans*-factors used for expression of crystallin genes in the lens and other tissues.

(2) Give particular attention to Pax-6 and prox1 transcription factors, especially with respect to their possible roles in crystallin gene expression.

(3) Continue investigations concerning the possible role(s) of α-crystallins in metabolic reactions such as the regulation of transcription.

(4) Continue the cloning and characterization of the cubomedusan jellyfish crystallin genes and their putative regulatory sequences.

(5) Continue to investigate the structure and possible functions of the cephalopod S-crystallins and the expression of their genes.

NEI Research Program

Lens and Cataract—Molecular Genetics

Publications

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Piatigorsky J: The twelfth Frederick H. Verhoeff Lecture: gene sharing in the visual system. *Trans Am Ophthalmol Soc* LXXXI: 283-298, 1993.

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West JA, Sivak JG, Pasternak J, Piatigorsky J: Immunolocalization of S-crystallins in the developing squid (*Loligo opalescens*) lens. *Dev Dynamics* 199:85-92, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00259-05 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of the Cornea

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Joram Piatigorsky	Ph.D.	Chief	LMDB, NEI
Others:	W. Todd Kays	Ph.D.	IRTA	LMDB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Molecular Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.05	1.05	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Aldehyde dehydrogenase class 3 (ALDH3) comprises approximately 40 percent of the cellular protein of the mammalian corneal epithelial cells, an amount reminiscent of an enzyme-crystallin in the lens. Consequently, we are investigating the molecular basis for the high expression of the ALDH3 gene in the corneal epithelial cells. The results will be compared with those obtained for crystallin genes in the lens and will provide a foundation for eventual gene therapy in the cornea. The complete mouse ALDH3 protein has been deduced from a cloned corneal complementary deoxyribonucleic acid (cDNA). Three 16-18 kbp mouse genomic fragments for ALDH3 have been cloned: one comprises the entire ALDH3 gene, one contains about 13 kbp of 5′ flanking sequence, exon 1, intron 1 (3.2 kbp) and part of exon 2, and the third is being analyzed. Northern blots have established that ALDH3 messenger ribonucleic acid (mRNA) is at least 100 times more prevalent in the cornea than in the stomach, bladder, and lung, the only other tissues showing a trace of this gene product. Transfection and transgenic mouse experiments using the chloramphenicol acetyltransferase (CAT) reporter gene have shown that 1050 bp of 5′ flanking sequence of the ALDH3 gene gives low-level expression in the liver, but is inactive in all other tissues tested, including the cornea. New promoter/CAT constructs containing intron 1 of the ALDH3 gene have been made and are being tested. The 5′ flanking sequence of the ALDH3 gene contains numerous potential control elements, including antioxidant response elements, which will be tested for function.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The project objectives are to identify and characterize the genes that are preferentially expressed in the epithelium and endothelium of the cornea and to study the molecular basis for their expression in this transparent eye structure.

Methods

Conventional molecular biology methods of cloning, sequencing, recombinant deoxyribonucleic acid (rDNA) construction, transfection, and transgenic mouse production are used.

Major Findings

Aldehyde dehydrogenase class 3 (ALDH3) comprises approximately 40 percent of the soluble protein of the corneal epithelial cells in the mouse and other mammals, including humans. Such abundance suggests that this metabolic enzyme may have a refractive function like an enzyme-crystallin in the lens. It is also possible that the high concentration of ALDH3 protects the eye from ultraviolet absorption and/or protects the cornea from oxidative damage. Moreover, the extremely high expression of the ALDH3 gene in the corneal epithelial cells provides a potential source of regulatory sequences for directing gene expression to this tissue, which could be of great value for gene therapy.

Last year, we created transgenic mice carrying a transgene comprising the β-galactosidase reporter gene fused to 1.1 kbp of the cloned mouse ALDH3 gene that we thought contained the promoter. However, no expression of the transgene was observed in any of the tissues of the transgenic mice that were examined, including the cornea. We thus performed rapid amplification of complementary DNA (cDNA) ends (RACE) experiments using corneal ribonucleic acid (RNA) and showed the existence of an additional 5′ exon, indicating that our construct lacked the corneal promoter. This 5′ exon does not appear to be present in the human ALDH3 gene on the basis of the published literature.

This year, we have characterized a new 16 kbp mouse ALDH3 genomic fragment that was shown to contain approximately 13 kbp of 5′ flanking sequence, exon 1 (40 bp), intron 1 (about 3.2 kbp), and part of exon 2 (40 bp). Two additional mouse ALDH3 genomic clones were obtained that were 16 kbp and 18 kbp, respectively. The 18 kbp fragment appears to contain the whole gene on the basis of sequencing with exon specific primers; the 16 kbp fragment is in the process of being analyzed. The entire mouse ALDH3 sequence was deduced from a full-length cDNA that we isolated from a corneal library.

The high expression of the ALDH3 gene in the mouse and bovine cornea was confirmed by Northern blot experiments. A trace of ALDH3 messenger RNA (mRNA) was present in the stomach, bladder, and lung (at least 100 times less than the cornea), and no ALDH3 mRNA was observed in the lens, adrenal gland (except a dubious smear of higher molecular weight), brain, and liver.

Constructs were made that contain the -1050/+21 fragment of the ALDH3 gene fused to the chloramphenicol acetyltransferase (CAT) gene in both the forward and backward orientation. Transfection tests using corneal SIRC cells and lens N/N1003A cells (both from rabbit) showed that this plasmid has low but detectable promoter activity leading to CAT expression when the putative ALDH3 promoter is in the correct orientation. Unexpectedly, however, this transgene did not express in transgenic mice, except for limited amounts in the liver. The cornea was painfully devoid of CAT in the transgenic mice. Consequently, new constructs have been made that contain 1050 bp of 5′ flanking sequence, exon 1, the 3.2 kbp intron 1 and the first 5 bp of exon 2 (excluding the ATG translation start codon) of the ALDH3 gene fused to the CAT gene to test the possibility that a corneal enhancer is present in intron 1. At the time of writing, these constructs are being tested for promoter

activity in transfected cells and will be used to generate transgenic mice.

Finally, analysis of the 5′ flanking sequence of the ALDH3 gene has revealed numerous potential regulatory elements, including NF-kB, AP-1, NFI, AP-2, SP-1, GRE, EivF/CREB, ARE, and XRE sites. The presence of ARE and XRE sites supports the idea that the high expression of this gene may be connected to an ultraviolet responsive or antioxidant response.

Significance to Biomedical Research and the Program of the Institute

The molecular biology of corneal epithelium and endothelium has not advanced to the same extent as that of the collagenous stroma; consequently, it should be investigated. The cornea is a transparent ectodermally derived tissue like the lens. Thus, comparative studies between it and the lens are of special interest with respect to transparency and the regulation of gene expression.

Moreover, because of our finding several years ago that corneal epithelial cells show taxon-specific gene sharing of metabolic enzymes, as does the lens, comparative studies on the cornea and lens are important from developmental and evolutionary perspectives. Finally, the cornea is particularly accessible for gene therapy on account of its exposure to the surface and its association with numerous hereditary diseases. The identification of a functional promoter with corneal specificity will open the door to gene therapy and genetic manipulation in the cornea as isolation of crystallin promoters with lens specificity did in that tissue.

Proposed Course

The following investigations are planned for fiscal year 1995:

(1) Identify the corneal-specific regulatory elements in the ALDH3 gene of the mouse by transfection and transgenic mouse experiments.

(2) Establish a convenient culture system to analyze the expression of ALDH3 and other corneal genes by transfection.

(3) Begin to analyze the *trans*-factors used for expression of the mouse ALDH3 gene in corneal epithelial and, perhaps, endothelial cells.

(4) Obtain a human ALDH3 gene for comparison with the mouse gene and to provide the foundations for gene therapy in human corneas.

NEI Research Program

Corneal Diseases—Structure and Function, Stroma

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00255-06 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology and Functions of Lens Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Graeme J. Wistow	Ph.D.	Chief, Section on Molecular Structure and Function	LMDB, NEI
Others:	Vishwas Paralkar	Ph.D.	Visiting Associate	LMDB, NEI
	Caroline Graham	B.S.	Biologist	LMDB, NEI
	Lorenzo Segovia	Ph.D.	Visiting Fellow	LMDB, NEI
	Jill Richardson	Ph.D.	Visiting Fellow	LMDB, NEI
	Ronit Frilling	Ph.D.	Visiting Fellow	LMDB, NEI
	Cynthia Jaworski	Ph.D.	Chemist, Section on Molecular Genetics	LMDB, NEI

COOPERATING UNITS *(if any)*

National Institute of Allergy and Infectious Diseases (Christine Kozak, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Molecular Structure and Function

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.9	5.9	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Crystallins are the major components of the normal eye lens. In a novel evolutionary process crystallins have arisen by the gene recruitment of stress-proteins and enzymes without prior gene duplication. In the lens-specific alternative promoter of guinea pig NADPH:quinone oxidoreductase/ζ-crystallin we have identified an element that is essential for function. We now find that this element contains a binding site for Pax-6, a "master gene" for eye development. Mutation of the Pax-6 site abolishes promoter function and Pax-6 is required for formation a lens-specific complex. Futhermore, Pax-6 is expressed in the mature lens. Pax-6, thus, has a continuing role in maintenance of lens-specific gene expression and a key role in the gene recruitment of a crystallin. μ-crystallin, which was discovered in marsupial lenses, is a novel NADPH-binding protein related to enzymes of ornithine and glutamate metabolism. In humans, in which it has not been recruited as a crystallin, it is expressed most abundantly in photoreceptor outer segments, suggesting an unexpected role in the visual process. Other proteins are essential for normal development in lens. Migration inhibitory factor (MIF), a small protein expressed in differentiating lens cells, is essential for progression through the cycle. Antisense to MIF abolishes proliferation of growth factor stimulated and cancer cells and halts them at the G1/S boundary. Promoter analysis of the human MIF gene has identified a region required for growth factor response. We have also found that LP2, a lipid/retinoid binding protein we have cloned from bovine lens, is expressed preferentially in differentiated fiber cells.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Ales Cvekl	Ph.D.	Visiting Fellow, MG, LMDB, NEI
Steven Puopolo		Summer Program

Objectives

We are investigating the molecular basis of normal lens structure and function. This includes the characterization of crystallins, their lens and nonlens function, and their mechanisms of expression and recruitment. This has entailed the identification of factors responsible for regulation of lens cell differentiation and gene expression. The interplay of such factors is an essential part of normal lens development and function.

Methods

A wide range of molecular biology techniques are used, including messenger ribonucleic acid (mRNA) analysis, gene and complementary deoxyribonucleic acid (cDNA) cloning and sequencing, functional gene promoter analysis in cultured cells and in transgenic mice, polymerase chain reaction, and antisense technology. We perform some protein analysis and make use of commercial facilities for protein sequencing. We make extensive use of computer-based sequence analysis and molecular modeling.

Major Findings

(1) Gene Recruitment: Enzyme Crystallins. Guinea pig ζ-crystallin is a taxon-specific crystallin, an enzyme that has achieved high expression in lens through an alternative, lens-specific promoter. This promoter contains an element (ZPE) that binds tissue-specific complexes in electrophoretic mobility shift assay. Jill Richardson has shown that both lens-specific complex formation and promoter function depend on an intact binding site for Pax-6 in the ZPE. Pax-6 is expressed in embryonic eye and central nervous system and is implicated in the establishment of lens competence. Antisera to Pax-6 specifically abolish lens complex formation by the ZPE. Protein and mRNA for Pax-6 are present in mature mouse lens and in cells that support ζ-crystallin lens promoter expression but not in other cell types. This suggests that ζ-crystallin is a target gene for Pax-6 and supports the idea that Pax-6 is a "master gene" factor for tissue-specific gene expression in the lens. Ronit Frilling has shown that the isolated fragments covering the ZPE region of the ζ-crystallin promoter can confer lens-preferred expression on a heterologous promoter, the thymidine kinase minimal promoter driving a reporter gene. Experiments in transgenic mice show that sequences upstream of the Pax-6 site, between -498 and -385, are required for suppression of promoter function in brain, which is also a site of Pax-6 expression.

Although some taxon-specific crystallins are familiar enzymes, others were discovered first as crystallins. μ-Crystallin is the major component of the eye lens in several Australian marsupials. In other mammals, including humans, it is expressed at enzymatic levels in lens, but Lorenzo Segovia has shown that it is most strikingly expressed in retinal photoreceptor cell outer segments. μ-Crystallin has sequence similarity with bacterial ornithine cyclodeaminases and glutamyl-transfer RNA reductases, suggesting a role in amino-acid metabolism that probably involves a glutamate-related pathway. This is particularly significant because glutamate is the neurotransmitter of the photoreceptors. The gene for μ-crystallin has been cloned from both kangaroo, where it has undergone gene recruitment, and from human, where it serves only as an enzyme. The human gene for μ-crystallin maps close to a locus for congenital cataract and microphthalmia. μ-Crystallin has also been mapped in the mouse genome.

Another major enzyme crystallin (up to 25 percent of total protein) in mammals is η-crystallin found in elephant shrews. Caroline Graham has cloned the complete cDNA sequence for this protein from two different species. Current results suggest that η-crystal-

lin is very closely related to aldehyde dehydrogenase 1 (ALDHI) and is expressed both in lens and liver. However, a second, closely related ALDH1 is also expressed in liver, suggesting that the gene recruitment of η-crystallin has led to gene duplication and specialization, perhaps as a consequence of adaptive conflict. The elephant shrew genome also contains several processed pseudogenes for η-crystallin/ALDH1-like sequences.

αB-crystallin is multifunctional, serving as both a major structural protein in the lens and as a small heat shock protein in other tissues in mammals. The gene for αB-crystallin in a bird (*Anas platyrhynchos*) has been cloned and sequenced. Because only the most important functional features of a gene are conserved among distantly related species, comparison of this sequence with those of mammals can be very informative. Although several functionally defined elements in mammals are conserved in the duck gene, consensus stress-response elements are not well conserved. Current experiments are aimed at determining whether the bird gene does indeed have the stress role suggested for mammalian genes.

(2) Molecular Markers of Differentiation. Macrophage migration inhibitory factor (MIF) was originally identified as a lymphokine. However recent work strongly suggests a role for MIF beyond the immune system. We have found that it is expressed specifically in the differentiating cells of the immunologically privileged eye lens and in many other tissues. Now Vishwas Paralkar has shown that MIF is an essential part of the mitogenic response to growth factors operating through two different receptor systems. Both platelet-derived growth factor and transforming growth factor β1 stimulate expression of MIF in NIH 3T3 cells in a delayed early response. When cells treated with either growth factor are grown in the presence of antisense oligo to mouse MIF, MIF protein synthesis and cellular proliferation are abolished. Control oligos have no effect under identical conditions. When antisense oligo is removed the cells regain the ability to proliferate in response to mitogenic signals. Analysis of levels of cyclins E, A, and B suggests that the effect of MIF is localized close to the G1/S transition in the cell cycle and that MIF is essential for progression into S phase. In promoter analysis of the human MIF gene a region required for induction by growth factors has been localized. This MIF gene region also contains an E-box, a potential *myc* family-binding site. The gene for MIF has been mapped in human and mouse.

LP2 is a member of the lipid/retinoic acid binding family of P2 proteins. Cynthia Jaworski has cloned bovine LP2 and has shown that its mRNA is expressed preferentially in differentiated fiber cells. Because retinoic acid has now been implicated in fiber cell specific γ-crystallin gene expression, this protein could have a direct role in mediating lens gene expression. Molecular modeling shows that LP2, unlike adipocyte P2, myelin P2 or other relatives, contains two close cysteine residues capable of disulfide cross-linking. LP2 is thus a potential target for oxidative damage to the lens in aging and cataract.

Significance to Biomedical Research and the Program of the Institute

We are uncovering fundamental mechanisms in the evolution and differentiation of the lens that may be generalizable to other complex tissues. We have shown how a single gene can have multiple functions and how it can acquire tissue-specific patterns of expression. In examining proteins that have been recruited as crystallins, we have discovered a novel enzyme that may have an important role in human retinal photoreceptors. Our work in lens has also revealed important markers for cellular differentiation. In particular one of these proteins, MIF, has an essential role in cell proliferation, and antisense treatment to suppress MIF halts proliferation, even of transformed cells.

Proposed Course

(1) To continue to examine the molecular mechanisms for lens preferred expression and gene recruitment of crystallins.

(2) To characterize the function and nonlens role of μ-crystallin, particularly its role in photoreceptor outer segments.

(3) To define the role of MIF in the cell cycle and to test the potential for antisense treatment to suppress the growth of transformed cells.

(4) To determine the function of LP2 protein, a marker for differentiation in the lens.

NEI Research Program

Lens and Cataract—Molecular Genetics

Patent

G.J. Wistow. US Patent 5,328,990: "Isolation of macrophage migration inhibition factor from ocular lens."

Publications

Graham C, Wistow G: The predominant cadherin in fetal human lens is identical to N-cadherin and is not a candidate locus for the Marner cataract. *Exp Eye Res* 59, in press.

Lee DC, Gonzalez P, Wistow G: Zeta-crystallin: a lens-specific promoter and the gene recruitment of an enzyme as a crystallin. *J Mol Biol* 236:669-678, 1994.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00251-07 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetically Engineering the Eye with the αA-Crystallin Promoter

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ana B. Chepelinsky	Ph.D.	Head, Section on Regulation of Gene Expression	LMDB, NEI
Others:	Devonne M. Parker	B.S.	Biologist	LMDB, NEI
	Charles Egwuagu	Ph.D.	Scientist, PHS	LI, NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
	Robert B. Nussenblatt	M.D.	Clinical Director	LI, NEI
	Jorge Sztein	D.V.M.	Visiting Associate	VRRS, NEI

COOPERATING UNITS *(if any)*

Department of Cell Biology, Baylor College of Medicine, Howard Hughes Medical Institute (Paul Overbeek, Ph.D.; Michael Robinson, Ph.D.); Imperial Cancer Research Fund, London, England (Clive Dickson, Ph.D.); Gerontological Research Unit, National Institute of Health and Medical Research, Paris, France (Yves Courtois, Ph.D.; Maryvonne Laurent, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Regulation of Gene Expression

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.5	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We generated transgenic mice and rats expressing interferon (IFN)-γ in their lenses to investigate the possible role of this lymphokine in ocular pathogenesis. Embryonic lens and retina differentiation were affected in the αA-crystallin/IFN-γ transgenic mice resulting in microphthalmia, microphakia, retinal detachment, and persistent hyperplastic primary vitreous in the adult mice. Major histocompatibility complex (MHC) class II messenger ribonucleic acid (mRNA) levels were significantly increased in the transgenic eyes, and MHC class II proteins were expressed in their cornea, iris, ciliary body, choroid, lens, and retinal pigment epithelial (RPE). Constitutive expression of IFN-γ and its induction of MHC class II molecules in the eye provide a useful model to study the linkage between aberrant MHC class II expression and predisposition to autoimmunity and the role of IFN-γ in the treatment of inflammatory eye diseases and cytokine signaling during embryonic eye development.

Fibroblast growth factor (FGF)-3 expression was directed to the eye to investigate how the aberrant expression of this growth factor would affect the developmental program of the eye. The αA-crystallin/FGF-3 transgenic mice presented exophthalmia and aberrant elongation of central lens epithelia at 15 days of embryonic development. The hypertrophic lens mass was extruded through the cornea at 16 days of embryonic development, resulting in cornea perforation. Postnatal microphthalmia was characterized by intraocular hyperplastic glandular structures replacing the normal iris, ciliary body, and lens.

Project Description

Objectives

The objective of this project is to understand how aberrant genetic expression of interferon gamma (IFN-γ) or fibroblast growth factor-3 (FGF-3) under the control of the αA-crystallin promoter perturbs normal eye development in transgenic mice.

Methods

Recombinant deoxyribonucleic acid (rDNA) techniques used in this study include plasmid construction, oligonucleotide sequencing, Southern and Northern hybridizations, DNA sequencing, primer extension, polymerase chain reaction (PCR), reversed transcription PCR. Other molecular biology techniques used in the study include immunohistochemistry, *in situ* hybridization, and production and analysis of transgenic mice.

Major Findings

(1) IFN-γ. This project is conducted in collaboration with Drs. Charles Egwuagu, Jorge Sztein, Chi-Chao Chan, and Robert B. Nussenblatt from the Laboratory of Immunology (LI) at the NEI. The IFN-γ gene is specifically expressed in activated T lymphocytes and natural killer cells. It plays a crucial role in the ontogenesis and phenomenology of the immune response and has potent immunomodulatory and antiproliferative effects on tumor cells. The ectopic expression of IFN-γ in the lens of transgenic mice allowed us to study its effect on eye development and its regulation of major histocompatibility complex (MHC) class II gene expression in a nonlymphoid tissue such as the lens.

We previously generated FVB/N and BALB/c transgenic mice containing as a transgene the murine αA-crystallin promoter (-366/+46) fused to the murine IFN-γ coding sequence. In both αA-crystallin/IFN-γ transgenic mouse lines, ectopic expression of IFN-γ in the lens affected the growth of the whole eye, resulting in microphthalmia and blepharophimosis. Lens differentiation was severely affected resulting in microphakia, impairment of lens fiber formation and cataract, thickening of the anterior lens capsule, and rupture of the posterior capsule. Retardation of retinal differentiation into inner and outer neuroblastic layers was observed in the transgenic eyes. Serous retinal detachment with presence of macrophages in the subretinal space, persistent hyperplastic primary vitreous, corneal vascularization, and absence of a normal anterior chamber were observed in the adult transgenic mice. MHC class II messenger ribonucleic acid (mRNA) levels were significantly increased in the eyes of transgenic mice, and MHC class II proteins were expressed in the corneas, irises, ciliary bodies, choroids, lenses and retinal pigment epithelia. Expression of genes coding for IFN-γ-inducible transcription factors, interferon-consensus-sequence-binding protein, and interferon response factor 2, absent in the normal eye, were induced in the transgenic eyes. These results indicate that the ectopically expressed transgenic IFN-γ is biologically active *in vivo.*

We also derived a transgenic Sprague Dawley rat line using the same murine αA-crystallin/IFN-γ construct. The transgenic rat eyes, similar to those from the transgenic mice, present microphthalmia and microphakia with cataract formation. However, in contrast to the transgenic mouse, the anterior chamber of the transgenic rat eye is well formed, and the architecture of the retina is intact with focal retinal serous detachment. The αA-crystallin/IFN-γ transgenic rat, the first transgenic rat strain generated for vision research, is of particular interest because the rat is a well-characterized species for experimental autoimmune uveitis studies.

(2) FGF-3. This is a collaborative project with Drs. Paul Overbeek and Michael Robinson, from Baylor College of Medicine, and Dr. Clive Dickson, from the Imperial Cancer Research Fund. FGF-3/int-2 is a member of the fibroblast growth factor (FGF) family. To assess whether ectopic expression of FGF-3 would perturb normal lens development, we

directed its expression to the eyes of transgenic mice using the murine αA-crystallin promoter. We obtained three lines of transgenic mice expressing the αA-crystallin/FGF-3 transgene. The anterior lens epithelia of the transgenic mice undergo premature cell elongation at day 14 of embryonic development concomitant with major intrinsic protein (MIP) synthesis. The lens mass was extruded through the cornea, resulting in cornea perforation at 16 days of embryonic development. At postnatal day four, intraocular glandular structures that replaced the normal irises, ciliary bodies, and lenses and stained positively for FGF-3 and Muc-1 (a marker for secretory epithelia) and negatively for connexin 46 and MIP were observed. We observed a marked increase in Muc-1 mRNA levels, while MIP, connexins 46 and 50 mRNA levels were drastically reduced in the adult transgenic mouse eyes. The ectopic expression of the FGF-3 during the embryonic development of the lens induces premature differentiation of the central lens epithelia, expulsion of the lens through the cornea, and postnatal appearance of intraocular secretory glandular epithelia.

(3) *Acidic FGF*. In collaboration with Drs. Overbeek and Robinson and Dr. Yves Courtois, from the Institute for Gerontological Research (INSERM), we obtained three lines of transgenic mice carrying as a transgene the αA-crystallin promoter fused to the bovine acidic FGF complementary DNA. These mice do not present any particular phenotype. We are currently studying whether the lens cells derived from these mice are able to give rise to a lens epithelia cell line with differentiated properties.

Significance to Biomedical Research and the Program of the Institute

Constitutive expression of IFN-γ, and its induction of MHC class II molecules in the eye, provides a useful model to study the linkage between aberrant MHC class II expression and predisposition to autoimmunity and the role of IFN-γ in the treatment of inflammatory eye diseases and cytokine signaling during embryonic eye development. The ectopic expression of FGF-3 will allow us to elucidate the mechanisms underlying eye development.

Proposed Course

The following studies will continue during fiscal year 1995:

(1) The effect of IFN-γ on the regulation of gene expression in the eyes of the transgenic mice will be further characterized.

(2) The effect of FGF-3 on gene expression in the eyes of transgenic mice will continue to elucidate the role of FGF-3 in premature lens epithelia differentiation and the appearance of intraocular secretory epithelia.

NEI Research Program

Lens and Cataract—Molecular Genetics

Publications

Chepelinsky AB, Robinson M, Overbeek PA, Parker DM, Chan C-C, Jamieson S, Dickson C: FGF-3 ectopic expression induces differentiation of central lens epithelia and appearance of secretory epithelia in the eyes of transgenic mice. *Invest Ophthal Vis Sci* 35(suppl):1988, 1994.

Egwagu CF, Sztein J, Chan C-C, Reid W, Mahdi R, Nussenblatt RB, Chepelinsky AB: Ectopic expression of gamma interferon expression in the eyes of transgenic mice induces ocular pathology and MHC class II gene expression. *Invest Ophthal Vis Sci* 35:332-341, 1994.

Egwagu CF, Sztein J, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Gamma interferon expression disrupts lens and retinal differentiation in transgenic mice. *Dev Biol*, in press.

Egwagu CF, Sztein J, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Transgenic rat and mouse models for the study of intraoc-

ular effects of IFN-γ and autoimmunity. *Invest. Ophthal Vis Sci* 35 (suppl):1987, 1994.

Egwagu CF, Sztein J, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Transgenic rat and mouse models for studying the role of gamma interferon and MHC class II in intraocular diseases and autoimmunity. *Proceedings of the 6th International Symposium on the Immunology and Immunopathology of the Eye,* in press.

Sax CM, Cvekl A, Kantorow M, Sommer B, Chepelinsky AB, Piatigorsky J: Identification of negative-acting and protein-binding elements in the mouse αA-crystallin -1556/-1165 region. *Gene* 144:163-169, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00253-06 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Expression of Lens Fiber Membrane Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ana B. Chepelinsky	Ph.D.	Head, Section on Regulation of Gene Expression	LMDB, NEI
Others:	Chiaki Ohtaka-Maruyama	Ph.D.	Visiting Fellow	LMDB, NEI
	Xiaoyan Wang	M.D.	IRTA Fellow	LMDB, NEI
	Devonne M. Parker	B.S.	Biologist	LMDB, NEI
	Chris Chon		Summer Student	LMDB, NEI

COOPERATING UNITS *(if any)*

Harvard University (David Paul, Ph.D.); Columbia University (Jorge Fischbarg, M.D.); Tokio Medical and Dental University, Japan (Sei Sasaki, M.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Regulation of Gene Expression

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.37	2.5	0.87

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project studies the regulation of expression of genes encoding lens fiber membrane channel proteins. We are presently focusing on the regulation of expression of the gene encoding, the major intrinsic protein (MIP) of the lens fiber membrane, that is specifically expressed in the ocular lens fibers and belongs to an ancient superfamily of transmembrane channel proteins.

We are studying the *cis* regulatory elements responsible for the lens specificity and developmental regulation of the MIP gene. We are fusing 5' flanking sequences of the human MIP gene to the reporter chloramphenicol acetyltransferase (CAT) gene and analyzing CAT gene expression in transient assays in primary cultures of lens cells. Deletion analysis of the 5' flanking sequence of the human MIP gene suggests that negative regulatory elements are located within the sequence -2840/-254. The human MIP sequence -70/-40 appears to contain an important regulatory element for the activity of the MIP promoter. This sequence contains one of the domains that interacts with Sp1 and AP2 transcription factors by DNase I footprinting analysis; it also forms a complex with chicken lens nuclear extracts that appear as a retarded band in mobility shift assays. Mutations at positions -49/-51 affect binding of this nuclear factor *in vitro*, and deletion of the sequence -70/-49 abolishes promoter activity in transfected lens cells. These studies will further our understanding of the regulation of the MIP gene expression in the lens.

Project Description

Objectives

The objective of this project is to elucidate the mechanisms involved in the regulation of expression of fiber membrane genes involved in cell-cell communication in the lens. The identification of the *cis* regulatory elements of these genes and their interaction with *trans*-acting factors are essential for understanding the regulation of gene expression in the lens.

Methods

Recombinant deoxyribonucleic acid (rDNA) techniques used in this study include screening genomic libraries; subcloning; plasmid construction; oligonucleotide synthesis; Southern and Northern hybridizations; DNA sequencing; primer extension; polymerase chain reaction (PCR); reversed transcription PCR; gel mobility shift assays; DNA footprinting; methylation interference; preparation of nuclear extracts by subcellular fractionation; *in vitro* transcription; tissue culture techniques, including transfection of primary lens explants and cell lines; analysis of transgenic mice; *in situ* hybridization.

Major Findings

(1) Cis Regulatory Elements of the Human Major Intrinsic Protein (MIP) Gene Promoter. We have characterized 2840 bp of 5′ flanking sequence of the human MIP gene to identify the *cis* regulatory elements responsible for the tissue specificity and developmental regulation of the MIP gene. We found that a DNA fragment containing the human MIP sequence -253/+42 fused to the reporter chloramphenicol acetyltransferase (CAT) gene activates CAT gene expression when transfected into lens cells. However, the -2840 /+42 sequence does not activate CAT gene expression, suggesting that negative regulatory elements are located within the sequence -2840/-254. Deletion mutants experiments indicated that -70/+42 sequence contains an active promoter in transfected lens cells. However, when sequences -70/-49 are deleted, the promoter activity is lost. The human MIP sequence -70/-40 appears to contain an important regulatory element for the activity of the MIP promoter. An oligonucleotide corresponding to sequence -67/-38 forms a complex with chicken lens nuclear extracts that appear as a retarded band in mobility shift assays. Mutations at positions -49/-51 affect binding of this factor. This sequence corresponds to one of the domains that interact with Sp1 and AP2 transcription factors by DNase I footprinting analysis.

We have generated two lines of transgenic mice containing 2840 bp of the human MIP gene 5′ flanking sequence and 42 bp of exon one fused to the β-galactosidase gene as a transgene. We are presently analysing β-galactosidase expression in these transgenic mice to determine whether *cis* regulatory elements responsible for lens-specific expression of the MIP gene are located in that domain.

(2) Cloning of the Mouse MIP Gene. We have isolated one MIP genomic clone from a P1 phage mouse genomic library. Subcloning and characterization of this clone will allow us to study the regulation of expression of the murine MIP gene.

(3) Cloning of the Connexin 46 Gene. The connexin 46 gene encodes one of the lens fiber gap junction proteins. To be able to study how its expression is regulated in the lens, we are sequencing, in collaboration with Dr. David Paul, the mouse connexin 46 gene from a clone isolated from a mouse genomic library.

(4) Aquaporin Gene Expression in Cornea Endothelial Cells. Although the physiological basis for the maintenance of corneal transparency has been extensively studied, the molecular mechanisms involved in maintaining cornea transparency are poorly understood. Swelling of the corneal stroma is involved in the loss of cornea transparency. In collaboration with Dr. Jorge Fischbarg, from Columbia University, we are characterizing the member of the MIP family responsible for the CHIP28-

like water channels observed in primary cultures of bovine cornea endothelial cells. Sequencing data indicate that it has a high degree of identity with CHIP28, suggesting that it may be a new member of the MIP family derived from CHIP28 by gene conversion.

Significance to Biomedical Research and the Program of the Institute

Proper lens fiber membrane biosynthesis and physiology are of upmost importance for maintaining lens transparency. Membrane protein synthesis is temporal and spatially regulated during lens development. Lens membranes undergo biochemical and structural changes during cataractogenesis and aging. Therefore, studying the regulation of genes encoding lens membrane channels should further our understanding, not only of the mechanisms involved in the regulation of gene expression in the normal lens but also of its disruption during disease.

Proposed Course

The following studies will continue during fiscal year 1995:

(1) Characterization of the *cis* regulatory elements of the mouse MIP gene promoter and comparison with its human homologue.

(2) Interaction of the mouse MIP gene *cis* regulatory elements with transcription factors.

(3) Sequencing of connexin 46 genomic clones to locate the 5′ end of this gene.

NEI Research Program

Lens and Cataract—Molecular Genetics

Publications

Chepelinsky AB: The MIP transmembrane channel family, in Peracchia C (ed): *Handbook of Membrane Channels.* San Diego, Academic Press, 1994, pp 413-432.

Chepelinsky AB, Ohtaka-Maruyama C, Wang X: General transcription factors and lens specific expression of the MIP gene. *J Cellular Biochem* 18(B):388, 1994.

Ohtaka-Maruyama C, Wang X, Chepelinsky AB: AP2 transcription factor is involved in the transcription of the lens MIP Gene. *J Cellular Biochem* 18(C):50, 1994.

Saito F, Sasaki S, Chepelinsky AB, Fushimi K, Marumo F, Ikeuchi T: Human AQP-2 and MIP genes, two members of the MIP family, map within chromosome band 12q13 using two-color FISH. *Cytogen Cell Genetics,* in press.

Wang X, Ohtaka-Maruyama C, Chepelinsky AB: *Cis* regulatory elements of the human MIP gene promoter. *Invest Ophthalmol Vis Sci* 35(suppl):1997, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00285-02 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

NEI Central Transgenic Animal Production Facility

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eric Wawrousek	Ph.D.	Research Biologist	LMDB, NEI
Others:	Susan DiCamillo	B.S.	Chemist	LMDB, NEI
	R. Steven Lee	B.S.	Biologist	LMDB, NEI
	Mariana Gonzalez-Baez		Biological Science Lab Aide, Stay-in-School Program	LMDB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Transgenic Animal and Genome Manipulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	0.5	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The NEI Central Transgenic Animal Production Facility is a research support facility for all NEI intramural researchers requiring the use of transgenic mice in their research programs. We are currently providing transgenic animal support to researchers from four laboratories in the NEI (Laboratory of Immunology, Laboratory of Mechanisms of Ocular Diseases, Laboratory of Molecular and Developmental Biology, and Laboratory of Retinal Cell and Molecular Biology). In our program, there are currently 84 deoxyribonucleic acid (DNA) constructs that are at various stages of completion. NEI researchers using molecular biology techniques to study the eye submit DNA constructs to our section for production of transgenic mice. We create transgenic mice by standard procedures, then biopsy and perform DNA analyses on the mice that are born from these procedures to identify positive mice. At researchers' request, we mate positive transgenic mice, wean litters, biopsy and analyze DNA from successive generations of transgenic mice, and provide the transgenic animals to researchers for use in their experiments. During the year, we have generated 155 transgenic founder mice from 34 DNA constructs; set up 456 matings of transgenic mice; weaned, tagged, and tail-biopsied 4,527 mice; isolated DNA from 4,708 samples; and performed 4,912 DNA analyses. This year we began an embryo cryopreservation and banking program to provide long-term storage of important transgenic lines without the need to maintain live mice. A total of 1,326 embryos from five transgenic lines have been frozen. In addition to service functions, we also collaborate with NEI researchers on transgenic animal projects.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

This project is to produce transgenic animals for use in eye research in the NEI, supply ancillary services related to maintenance of transgenic animals, and provide advice and expertise in matters of transgenic animal projects to all NEI intramural researchers using this technology in their research. In addition, we act as a central facility for all transgenic animal work conducted in the NEI intramural research program to coordinate and conserve resources and use severely limited animal housing space with maximum efficiency. We provide a comprehensive program for short- and long-term storage of transgenic animal lines for both live animals and frozen embryos.

Methods

Standard methods are used for microinjecting deoxyribonucleic acid (DNA) into the pronucleus of one-celled mouse embryos and surgically reimplanting the injected embryos into foster mothers to enable development. Conventional molecular biology techniques are used to isolate and analyze DNA from biopsy samples of transgenic mice. Data on all transgenic mice are maintained in a computerized, relational database accessed by programs written within our group.

Major Findings

Production of New Transgenic Mouse Lines. We have generated 155 new transgenic founder mice from 34 constructs submitted by researchers in four NEI intramural laboratories. These constructs are quite diverse in nature and reflect the diversity of research being performed in the NEI. Some of the general categories of constructs are: (1) promoter/reporter constructs in which the promoter of an eye gene is fused to a reporter gene to assess transcriptional activity in the transgenic mouse, (2) eye-specific or ubiquitous promoters driving expression of genes believed to be involved in eye pathologies to assess their roles in pathological conditions in a transgenic mouse model, and (3) other constructs for probing normal eye function and pathological conditions in the mouse.

Maintenance of Transgenic Mouse Lines. Transgenic mouse lines are derived by mating of the original transgenic founder mice and derivation of successive generations of progeny, which are then used in biomedical research. To generate lines of transgenic mice from our transgenic founder mice, we have set up 456 mouse matings and weaned, tagged, and biopsied 4,527 mice resulting from matings and microinjection procedures.

DNA Analyses. Approximately 15 to 30 percent of mice born from microinjected embryos are transgenic, and approximately 50 percent offspring from a transgenic mating are transgenic sensitive. A rapid, efficient, and reliable method of identifying transgene positive and negative mice is in place in our group. We have processed 4,708 biopsy samples to obtain DNA and performed 4,912 analyses to determine whether the mice were transgene positive.

Embryo Cryopreservation and Banking. We have begun freezing mouse embryos for banking of important lines of transgenic mice. Between 200 and 300 embryos must be frozen for each line of mice banked. This year, we have frozen and banked 1,326 embryos from five lines of transgenic mice. Successful reconstitution of these transgenic lines has been accomplished by thawing a small portion of the banked embryos and transferring them into the oviducts of pseudopregnant foster mothers.

Significance to Biomedical Research and the Program of the Institute

Transgenic mice are currently the only readily attainable system for studying gene expression in the context of an entire, intact animal. Although tissue culture can yield a great deal of information in many studies, a true understanding of how a particular gene affects an

organ (such as the eye) or an entire organism can only be obtained by studying that gene in the intact organism. We play a pivotal role in many NEI intramural research projects by providing the technology and expertise to insert into the mouse genes related to normal eye development and pathological conditions of the eye.

Proposed Course

(1) Continue producing new transgenic mice for NEI researchers as required for their research projects.

(2) Continue breeding and maintaining existing transgenic mouse lines needed for ongoing research in the NEI.

(3) Continue freezing and banking embryos from important lines of transgenic mice. This will free some of our limited animal housing space and ensure that important lines of mice will not be lost due to aging and loss of fertility.

NEI Research Program

Lens and Cataract—Molecular Genetics

Publications

As a service organization, we are generally not included as authors on publications resulting from research performed on the transgenic animals we produce and maintain.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00286-02 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

α-Crystallin Gene Disruption in the Mouse

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eric Wawrousek	Ph.D.	Research Biologist	LMDB, NEI
Others:	James P. Brady	Ph.D.	IRTA Fellow	LMDB, NEI

COOPERATING UNITS *(if any)*

University of Maryland Medical School (Nicholas Ambulos, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Transgenic Animal and Genome Manipulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The α-crystallins compose a large fraction of the soluble protein in the vertebrate lens, where they are believed to function as structural proteins; are the first crystallins to be expressed in the developing mouse lens; and are a relatively small family of crystallins encoded by only two genes, the αA- and αB-crystallin genes. The α-crystallins exhibit molecular chaperon activity and kinase activity and, at least in the case of αB-crystallin, have been shown to be expressed in a variety of nonlenticular tissues, where their function is unknown. Toward understanding the role of the α-crystallins in lens and nonlens tissues, we are attempting to functionally delete α-crystallins by disrupting their genes in mice. We are using the technique of homologous recombination in pluripotent mouse embryonic stem cells followed by generation of chimeric mice containing the altered stem cells. We have isolated and mapped 15 kb clones containing the αA- and αB-crystallin gene loci from a mouse 129SV library (the same strain as most of the embryonic stem cell lines currently in use). Construction of αA-crystallin, αB-crystallin, and αA-CRYBP1 knockout vectors is complete. We are mastering the technologies to (1) effect homologous recombination in embryonic stem (ES) cells and (2) introduce ES cells into embryos that develop into chimeric mice. In collaboration with Nicholas Ambulos (University of Maryland Medical School), we are sequencing the mouse αA-crystallin gene locus.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Ellen Liberman	Ph.D.	Anterior Segment Disease Branch of the Extramural Research Program, NEI
Christina Sax	Ph.D.	Senior Staff Fellow, LMDB, NEI

Objectives

The objective of this project is to disrupt the α-crystallin genes (αA and αB) in the mouse to study their effect on normal lens and eye development. Disruption of the genes will essentially delete these proteins from the mouse and enable us to analyze the effects these proteins have on expression of other lens proteins, developmental regulation and morphology of the lens and other eye structures, and the role of these proteins in nonlenticular tissues.

Methods

Standard molecular biology techniques were used to clone the α-crystallin genes from a mouse 129SV genomic library and construct "gene knockout" vectors. Disruption of the genes will be accomplished by the now standard technology of homologous recombination in pluripotent mouse embryonic stem cells, followed by insertion of the genetically altered cells into blastocyst mouse embryos to generate chimeric mice with the gene disruption. Chimeric knockout mice will be bred to generate mice with heterozygous and homozygous knockout mutations.

Major Findings

Gene Knockouts. Construction of αA-crystallin, αB-crystallin, and αA-CRYBP1 gene knockout vectors has been completed. These vectors contain large pieces of the respective gene functionally disrupted by insertion of the selectable marker for neomycin resistance and flanked by the negative selectable marker, HSV tk. Use of these positive and negative selectable markers to facilitate selection of appropriately modified cells is currently standard procedure in this field. Following electroporation of the αA-crystallin knockout vector into J1 mouse embryonic stem cells and selection with G418 and ganciclovir, approximately 200 colonies were picked. PCR and Southern blot analysis of these clones to detect correct homologous recombination is currently under way. Preliminary analysis indicates that 11 of the first 96 clones screened contain the appropriate αA-crystallin gene knockout.

The technology for incorporating the modified cells into intact mice is being mastered in our laboratory. We have successfully generated chimeric mice by microinjecting unmodified ES cells into mouse blastocysts and allowing the embryos to develop in pseudopregnant foster mothers. Several chimeras have been born in which ES cells contribute 10 to 90 percent of the cells in the mouse (estimated by coat color of the mice). We are also developing the newer method of creating chimeric mice by simple aggregation of morula stage embryos with clumps of ES cells.

Sequence of the Mouse αA-crystallin locus. Approximately 12.7 kb of the 15 kb αA-crystallin locus has been sequenced on both strands.

Functional Significance of Sequences Flanking the Mouse αA-Crystallin Gene. We are constructing vectors containing portions of the αA-crystallin gene locus to search for possible regulatory elements located far from the promoter region. A basic promoter vector containing the mouse αA-crystallin promoter (-366 to +46) fused to the bacterial chloramphenical acetyltransferase gene (CAT) reporter gene has been constructed. In transient transfection assays with the N/N1003 rabbit lens epithelial cell line, this construct elicits significantly higher levels of CAT activity than the corresponding promoterless vector.

Several large pieces of the αA locus have been inserted into the basic promoter, and

after completion of several additional constructs, all of the constructs will be tested in the transient transfection assay for modulation of promoter activity.

Significance to Biomedical Research and the Program of the Institute

Deletion of the α-crystallin proteins, individually or together, will provide a fundamental understanding of how these proteins function during normal lens development and how they may influence the structure and function of the lens and the entire eye. Additionally, it would give us insight into the function of these proteins in nonlenticular tissues that in turn could help us understand some of their more subtle roles in the eye.

Proposed Course

(1) Continue the gene knockout experiments in embryonic stem cells with the two additional knockout vectors (αB-crystallin and αA-CRYBP). Produce chimeric mice from appropriately modified ES cells for each of our three selected genes, and mate these mice to produce lines of knockout mice for investigation. Deletion of a single allele of either αA or αB will be useful in assessing gene dosage effect (50 percent reduction of protein), and breeding to homozygosity (deletion of both alleles) will allow us to study the consequences of complete absence of the individual protein. It will be extremely interesting to mate eventually αA and αB knockout mice to produce mice which are totally devoid of α-crystallin.

(2) Continue collaborative sequencing the αA-crystallin gene locus. Although a considerable amount is known about regulation of the mouse αA-crystallin gene, the complete sequence of the gene has not yet been determined. The complete sequence of the gene and flanking regions will be beneficial for designing and interpreting experiments with this gene.

(3) Continue construction of vectors that will be used in transient transfection assays to locate regulatory elements spatially distant from the promoter of the αA-crystallin gene. This along with the sequence of the locus will help to identify potential sites influencing levels of gene expression.

NEI Research Program

Lens and Cataract—Lens Development and Aging

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00291-01 LMDB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transgenic Animal Models

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eric Wawrousek	Ph.D.	Research Biologist	LMDB, NEI

COOPERATING UNITS *(if any)*

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Laboratory of Molecular and Developmental Biology

SECTION

Section on Transgenic Animal and Genome Manipulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Transgenic mice offer a unique tool for studying normal and pathological states associated with expression, overexpression, or misexpression a particular gene. Aberrant expression of genes believed to be involved in disease can be easily achieved with the well-established transgenic methodologies. The resulting transgenic mice often may be used as models for studying diseases associated with the respective gene. We have developed a line of transgenic mice that expresses a modified form of mature human interleukin (IL)-1β in the ocular lens. The afflicted mice exhibit a progressive inflammation of the eye and neovascularization of many eye tissues resulting in the eventual destruction of the eye. IL-1β messenger ribonucleic acid (mRNA) and protein have been detected at high levels in the eye. There is upregulation of vascular adhesion molecules and significant influx of inflammatory cells, predominantly macrophages. A systemic unresponsiveness to IL-1-mediated events has been observed in these mice.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Chi-Chao Chan	M.D.	Chief, Immunopathology Section, Immunology Section, NEI
Igal Gery	Ph.D.	Chief, Experimental Immunology Section, NEI
James Lai		Howard Hughes Medical Institute

Objectives

The objective of this project is to create transgenic mouse models of ocular disease by aberrantly expressing proteins believed to be involved in maintenance of normal state or initiation and potentiation of a pathological condition. Our first model involves expression of human interleukin (hIL)-1β in lens of transgenic mice to generate an abundant supply of identical mice afflicted with an ocular inflammatory disease of defined origin. These mice can be used to study the many parameters associated with progression of the inflammation and test therapeutic regimens for controlling the inflammation and consequent ocular damage.

Methods

Standard methods were used to construct the transgene with the murine αA-crystallin promoter driving expression of a cassette containing the human tissue plasminogen activator secretion signal fused in frame to the coding region of mature human IL-1β. Transgenic mice were made by microinjecting the transgene construct into FVB/N single cell embryos. Mice used in this study were F_1 hybrids of the transgenic FVB/N and DBA/2. These mice are heterozygous for a congenital retinal degeneration found in the FVB/N strain and have histologically normal retinas.

Major Findings

One line of transgenic mice was generated containing the IL-1β construct. This line contains a single complete copy of the transgene. Large amounts of hIL-1β have been detected in the eyes of these mice both at the messenger ribonucleic acid (mRNA) and protein levels but has not been detected in other tissues (mRNA by Northern analysis) nor in serum (protein by enzyme-linked immunosorbent assay). The afflicted mice exhibit a progressive ocular inflammation accompanied by neovascularization, resulting in the destruction of the eyes in adult mice. The inflammatory infiltrate consists predominantly of macrophages with some polymorphonuclear neutrophils and lymphocyte involvement. Increased expression of the vascular adhesion molecule intercellular adhesion molecule 1 was evident in eyes of these mice. Nontransgenic litter mates exhibit none of these characteristics. The transgenic mice are otherwise healthy exhibiting normal reproductive capacity and longevity.

A systemic unresponsiveness to IL-1 mediated events was observed in the transgenic mice. They showed a decreased susceptibility to the toxic effects of lipopolysaccharide injection. Injection of 40μg per gram body weight resulted in lethality in only 6.3 percent of transgenic mice compared with 86 percent lethality in nontransgenic mice. Thymocytes isolated from the transgenic mice were also less responsive to IL-1 in culture than those isolated from normal litter mates. The mechanism by which this systemic effect is induced is currently unknown.

Significance to Biomedical Research and the Program of the Institute

This model is the first instance of successful creation of a transgenic mouse containing the potent cytokine IL-1. This model provides a

readily accessible stock of identical mice exhibiting a consistent pattern of ocular inflammation beginning with nearly normal eyes at four days of age, progressing with age, and resulting in destruction of the eye in adults. The roles of other cytokines, cellular adhesion molecules and arachidonic acid metabolites in progression of the ocular inflammation can be studied as can the establishment of the systemic unresponsiveness to IL-1.

Proposed Course

(1) Continue characterization of the IL-1β transgenic line. Quantitate mRNA levels for mouse IL-1, IL-1 receptor and receptor antagonist in the eyes of affected mice. Study alterations in cytokine expression patterns and arachidonic acid metabolites in these mice.

(2) Attempt to ameliorate the inflammation in this model by administration of anti-inflammatory drugs or antibodies to cytokines or cellular adhesion molecules.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

LABORATORY OF OCULAR THERAPEUTICS

Report of the Chief
Laboratory of Ocular Therapeutics

Peter F. Kador, Ph.D.

The Laboratory of Ocular Therapeutics (LOT) focuses on the development, evaluation, and mechanism of action of new ophthalmic drugs to treat eye diseases. The LOT research team is examining aldose reductase inhibitors (ARI) and anticataract agents. In pursuing the development of more effective and less toxic ARIs, the efforts are progressing the development of an inhibitor unrelated to previous ARIs. At present, a new inhibitor is being developed using biochemical, pharmacological, and computer molecular design techniques. Studies designed to elucidate the specific mechanism(s) of how ARIs diabetic complications are also being conducted. In studies using galactose-fed dogs, LOT investigators have established that retinal changes associated with diabetic retinopathy progressed to the proliferative stage and that the dog represents the first animal model to demonstrate clinical and histological changes found in all stages of retinopathy. Studies are now focused on the development of proliferative retinopathy in long-term galactose-fed dogs. Biochemical changes observed in these *in vivo* studies are being investigated using *in vitro* tissue culture techniques. Biochemical turnover in these cells is being monitored through nuclear magnetic resonance. Magnetic resonance imaging techniques are also being used to measure *in vivo* ARI efficacy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00003-21 LOT

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pharmacology of Ocular Complications

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Peter F. Kador	Ph.D.	Chief	LOT, NEI
Others:	Ping Ding	Ph.D.	Visiting Fellow	LOT, NEI
	William Greentree	D.V.M.	IRTA	LOT, NEI
	Petra Lachner	D.V.M.	IRTA	LOT, NEI
	Yong Lee	Ph.D.	Staff Fellow	LOT, NEI
	Martin Lizak	Ph.D.	Staff Fellow	LOT, NEI
	Heike Neuenschwander	M.D.	Special Volunteer	LOT, NEI
	Irina Obrosova	Ph.D.	Visiting Scientist	LOT, NEI
	Libaniel Rodriguez	Ph.D.	Staff Fellow	LOT, NEI
	Katsumi Sugiyama	Ph.D.	Staff Fellow	LOT, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Ocular Therapeutics

SECTION

INSTITUTE AND LOCATION
NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 7.69	PROFESSIONAL: 7.69	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Events leading to the onset of various ocular complications are being investigated. Specifically, the role of the enzymes aldose reductase and aldehyde reductase in the onset and progression of retinopathy, cataract, keratopathy, pupil function changes, and iris and ciliary process structure changes associated with diabetes and galactosemia are being studied. In addition, methods for either delaying or preventing the onset and progression of these complications through the pharmacological control of these enzymes are being developed.

Events leading to the formation of several types of cataracts are also being investigated, as well as methods for controlling the onset of these cataracts through pharmacological intervention.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Robert Balaban	Ph.D.	National Heart, Lung, and Blood Institute
Duane Miller	Ph.D.	University of Tennessee College of Pharmacy, Memphis, Tennessee

Objectives

To gain insight into the mechanisms by which polyol-induced ocular diabetic complications and cataracts are formed and to develop methods for their regulation.

Methods

Diabetes can be experimentally induced in animals by injecting streptozotocin. Diabetes-related complications linked to the sorbitol pathway can also be induced in animals such as rats and dogs by feeding them a galactose-enriched diet. Cataract formation and clinical retinal changes in experimental animals can be monitored through fundus photography. Biochemical studies used for the purification of enzymes include column chromatography, polyacrylamide gel electrophoresis, isoelectric focusing, chromatofocusing, and high-pressure liquid chromatography. Polyol levels were determined by gas-liquid chromatography. Immunological analyses include the use of enzyme-linked immunosorbent assay, radioimmunoassay, Western blots, and immunohistochemical techniques using the coupled antibody DAB-PAP technique. Computational methods for enzyme analysis, inhibitor structure-activity-studies, and pupil-function changes require the use of the National Institutes of Health (NIH) PROPHET computer system and Charm and Quanta Computer Systems from Molecular Design.

Major Findings

Biochemical Studies. Studies on defining the inhibitor site of aldose reductase and aldehyde reductase and the development of new aldose reductase inhibitors (ARI) are continuing. To facilitate the rational development of more specific and potent ARIs, more specific knowledge of the structural and pharmacophoric requirements of the site at which ARIs interact is required. It has recently been reported that cocrystallization of human placental aldose reductase with the inhibitor zopolrestat resulted in a complex where the inhibitor was almost completely sequestered in the large, deeply elliptical hydrophobic pocket that forms the substrate site. Zopolrestat's observed location, which makes the active site pocket inaccessible to solvent or further productive binding of substrate, does not support structure activity relationships (SAR), observations, and kinetic results, which indicates that ARIs such as zopolrestat are either noncompetitive or uncompetitive inhibitors. Using an 5-iodoacetamido analog of the alrestatin as an affinity-labeled ARI, we have located an alternative site of interaction on aldose reductase. The affinity-labeled ARI was irreversibly bound to purified rat lens aldose reductase, and the bound residues were identified by mass spectrometry.

Modeling studies of the identified site were subsequently conducted on the protein structure of aldose reductase, calculated by Charm force field using the crystal structure coordinates of human placental aldose reductase published in the Brookhaven Protein Data Bank. Potential interactions of inhibitors at this site were estimated through docking and binding studies using Quanta 3.3. The apparent ARI site is composed of a single region independent of the substrate binding site. It contains a number of pharmacophoric elements previously proposed for the inhibitor site. These include the amino acids arginine, serine, and tyrosine; proline; and a lipophilic area containing hydrogen bonding substituents. Multiple three-point attachments that include possible nucleophilic interactions with either serine or tyrosine are possible. Also present are two carboxylate binding groups that can orient the inhibitor on the lipophilic binding site. This proposed inhibitor site contains a number of pharmacophoric ele-

ments previously proposed for the inhibitor site, and its location and composition are consistent with reported kinetic data, SAR observations, stereochemical requirements, and quantum chemical calculations.

Several reports suggest that a metabolite of S-88-0773 (4-[4-N,N,-Dimethylsulfamoyl)-piperazino]-2-methylpyrimidine) increases sugar alcohol levels in normal and diabetic rats and, as a result, speeds up the appearance of the polyol-induced complications. Others report that the compound slows down the appearance of complications by altering redox states of NAD(P)-couples through inhibition of sorbitol dehydrogenase. We have used this compound to investigate its effect on cataract formation. For these studies, young (50g) streptozotocin-induced diabetic rats received normal diet with or without 0.06 percent S-88-0773 while similar-aged nondiabetic rats received either normal, 20 percent, 30 percent, or 50 percent galactose diets with or without 0.06 percent S-88-0773.

Cataract progression was monitored weekly by portable slit-lamp microscopy. Animals were periodically killed and polyol levels in the lenses evaluated. Polyol levels were increased in both galactosemic and diabetic rats, and no difference in lens polyol levels between each corresponding group of rats treated with or without drug was observed in the galactose-fed groups. Increases in tissue polyol levels associated with sorbitol dehydrogenase inhibition were not anticipated in galactose-fed rats because galactitol is not metabolized by sorbitol dehydrogenase. Nevertheless, sugar cataract formation was delayed in both diabetic and galactose-fed rats treated with S-88-0773. These results suggest that S-88-0773, or its active metabolite, delays sugar cataract formation through a biochemically unknown mechanism not related to either sorbitol dehydrogenase or ARI.

Magnetization transfer contrast (MTC) enhancement, which generates high-contrast images based on tissue characteristics resulting from the interaction of water and macromolecules, was applied to document *in vivo* cataract formation and other structural changes associated with diabetic eye complications. For these studies, male beagles ranging from six to 24 months of age were fed a diet containing 30 percent galactose. All dogs were then placed in a General Electric 2-T Omega magnetic resonance imaging system under anesthesia with muscle relaxant at four-week intervals. M_s and M_0 images were acquired using gradient-recalled-echo sequences, with and without the saturation pulses, respectively, consisting of rf-irradiation 10 kHz off-resonance from the free-water proton signal. The T_{1sat} images were calculated from the data using a one-shot T_1-imaging sequence.

The acquired images were compared with photographs obtained with slit-lamp microscopy and retroillumination photography. Excellent images detailing the fine structures of the anterior segment that includes the lens, cornea, iris, ciliary body, choroid membrane, and Schlemm's canal were obtained by MTC. In these images the progression of osmotic cataract formation in the galactose-fed dogs could be followed from the initial appearance of distinct cortical vacuoles. Distinct fluctuations in lens size and shape were observed as lenses progressed to the more advanced cataractous stages.

^{19}F NMR spectroscopy is also being used to measure *in vivo* aldose reductase activity in the dog lens by measuring the conversion of 3-deoxy-3-fluoroglucose to 3-deoxy-3-fluorosorbitol. This work is an extension of the *in vivo* evaluation of aldose reductase activity in rabbit lenses. Initial spatial coordinates for lenses are calculated from ^1H-images determined on a 2.0 Tesla GE Omega-CSI spectrometer. The SLOOP-technique (Spectral Localization with Optimal Pointspread function) is then used with a proton decoupler to measure the accumulation of sorbitol in the rabbit lens. A double spin-echo sequence is used with selective excitation and refocusing pulses and with optimized phase-encoding gradient pulses using one-second repetition times and 25 millisecond echo times. SLOOP experiments indicate that 3-deoxy-3-fluorosorbitol can be observed in spectra of the anterior

portion of the lens when adequate amounts of 3-deoxy-3-fluoroglucose are administered.

Retinal Studies. Vascular changes associated with diabetic retinopathy can be experimentally produced in beagles fed a 30 percent galactose diet. In studies designed to clarify the initiating lesions and progression of diabetic retinopathy, we have documented the progression of retinal lesions from background through the proliferative stage in the dog with ophthalmoscopic, fluorescein angiographic, and histopathologic findings. Initial retinal changes include aldose reductase-linked formation of pericyte ghosts and subsequent development of acellular capillaries, microaneurysms, and intraretinal hemorrhages. This early retinopathy progresses to include the appearance of occluded vessels, areas of nonperfusion, and intraretinal microvascular abnormalities. Finally, proliferative retinopathy develops, including the formation of fibrovascular membranes seen histologically on both the retinal surface and the posterior hyaloid membrane.

Diabetes-like microvascular changes in galactosemic beagles have been shown to be arrested in a dose-dependent manner by ARIs. To determine if retinal changes also can be reduced through the marked reduction of galactitol production after early retinal lesions have developed, galactose diet was removed after either the appearance of pericyte ghosts or microaneurysm formation. The subsequent progression of retinal changes over 24 months was then quantitively compared with retinal changes in remaining galactose-fed dogs. For these studies, 10 control dogs were fed a normal diet, while 50 dogs were fed diet containing 30 percent galactose. The galactose diet was removed from 15 dogs after 24 months, at which time pericyte ghosts had developed; another 15 dogs were removed after 31 months when microaneurysms had developed. Eighteen remained on galactose diet. Beginning at 24 months, four to five eyes from each experimental group and two to three eyes from the control group were enucleated at six-month intervals. Isolated retinas were quantified as previously described (*J Ocular Pharmacol* 9:257, 1993). Significant increases in the endothelium/pericyte ratio and decreases in pericyte density were observed with the duration of galactose-feeding. Although no reversal of retinal lesions occurred, differences in the progression of retinal lesions between the galactose-fed and galactose-removed groups became evident after 12 to 24 months. This study suggests that reduction of polyol accumulation at the initial stages of background retinopathy beneficially reduces the progression of retinal lesions.

Corneal Studies. Specular microscopic studies indicate that the size (polymegathism) and shape (pleomorphism) of the hexagonal corneal endothelial cells change in diabetics. Similar morphometric changes of the corneal endothelium have also been experimentally observed in diabetic rats as well as in diabetic and galactose-fed dogs, and concomitant administration of ARIs can reduce these morphological changes. The purpose of this study was to examine whether corneal endothelial changes in galactose-fed dogs are reversible by the marked reduction of galactitol production after stopping prolonged galactose feeding. Ten control dogs were fed a normal diet, while 48 dogs were fed a diet containing 30 percent galactose. The galactose diet was removed from 15 dogs after 24 months, at which time pericyte ghosts in the retina had developed; another 15 dogs were removed after 31 months when retinal microaneurysms had developed. Eighteen dogs remained on galactose diet throughout the study.

Specular microscopy was conducted on members of all groups after 38 months of study, and the photographs were analyzed in masked fashion on the Bambi image analysis systems. The evaluation of the corneal endothelial cells revealed significant differences in the cell size and density among all galactose-fed dogs and normal, age-matched control dogs. Corneal endothelial changes were not significantly reduced in dogs fed galactose for either 24 or 31 months and then receiving normal diet for 14 and seven months, respec-

tively, indicating that amelioration of endothelial cell changes requires therapy before the advent of endothelial morphologic changes.

Significance to Biomedical Research and the Program of the Institute

Loss of vision from cataract and diabetic retinopathy is significant; therefore, methods for the pharmacological control of these ocular complications are required. We have developed an animal model that demonstrates advanced retinal vessel changes that are virtually clinically and histologically identical to those observed in advanced diabetic retinopathy. Our present studies in dogs demonstrate for the first time that loss of retinal pericytes, associated with aldose reductase, initiates retinal changes associated with both background and advanced diabetic retinopathy and that administration of ARIs in prevention studies can ameliorate the loss of pericytes and subsequent development of microaneurysms and retinal hemorrhages in a dose-dependent manner. The successful development of noninvasive methods for monitoring aldose reductase activity by nuclear magnetic resonance procedures may directly affect ongoing and planned clinical trials where this procedure could serve as a quantitative indicator of drug efficacy. Cataract is also one of the major causes of blindness in the developing world. In addition, loss of vision due to cataract is one of the major health problems of both the diabetic and the aging populations in the United States.

Proposed Course

These studies will be continued. Discovered ARIs will be pharmacologically evaluated and developed. The inhibitor site will be further probed through the use of affinity labels so that more potent and specific inhibitors may be developed. Studies on the mechanisms through which aldose reductase induces diabetic complications in various tissues will be continued.

NEI Research Program

Retinal Disease—Diabetic Retinopathy, Sickle Cell Retinopathy, and Other Vascular Abnormalities

Lens and Cataract—Pathogenesis of Cataract

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00275-03 LOT

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

NADPH Reductases and Polyol Pathway in Ocular Complications

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Sanai Sato	M.D., Ph.D.	Visiting Scientist	LOT, NEI
Others:	Peter F. Kador	Ph.D.	Chief	LOT, NEI
	E. Filippo Secchi	Ph.D.	Fogarty Fellow	LOT, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Ocular Therapeutics

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.05	2.05	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In diabetes the increased flux of glucose into the polyol pathway results in the accumulation of the sugar alcohol sorbitol that is linked to the onset of various diabetic complications, such as cataract formation and retinopathy. The sugar alcohol formation and properties of NADPH-dependent reductases in fibroblasts, the key cells in the formation of fibrous proliferative tissues, has been investigated.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

Glucose is metabolized into fructose through sugar alcohol sorbitol in sorbitol pathway. In diabetes the increased flux of glucose into the polyol pathway results in the accumulation of sugar alcohol sorbitol. Experimental evidence has demonstrated the link between the accumulation of sugar alcohols and the selective loss of pericytes, the initial lesion of diabetic retinopathy. In the more advanced stages of retinopathy, fibroblasts play a key role in the formation of fibrous proliferative tissues. This project is designed to investigate whether the sugar alcohol accumulation is also linked to the formation of proliferative tissues.

Methods

Biochemical techniques include affinity chromatography, chromatofocusing, isoelectric focusing, electrophoresis, immunoblot and gas chromatography for sugar analysis. ^{19}F NMR with 3-deoxy-3-fluoro-D-glucose (3FDG) is used to investigate the metabolism of glucose. The National Institutes of Health (NIH) Prophet computer system is used for kinetic analysis and IC50 calculations.

Major Findings

The crude extract of murine fibroblast cell line L929 cells displayed reductase activity with DL-glyceraldehyde as substrate and dehydrogenase activity with D-sorbitol as substrate, suggesting that these cells contain two enzymes of the polyol pathway: aldose reductase (and/or aldehyde reductase) and sorbitol dehydrogenase. A nuclear magnetic resonance study with 3FDG confirmed the conversion of glucose into fructose through sorbitol in mouse fibroblast cell line L929. Sugar alcohol accumulated in the cells cultured in medium containing 30 mM galactose, and this accumulation was inhibited by aldose reductase inhibitors (ARI). Purification studies demonstrated that the dominant reductase in L929 cells was aldehyde reductase rather than aldose reductase. Two other fibroblast cell lines established from dog and human also appeared to possess aldehyde reductase as the dominant reductase in these cells.

These results confirm the presence of the polyol pathway in three different fibroblast cell lines. However, aldehyde reductase rather than aldose reductase predominates in these cells.

Significance to Biomedical Research and the Program of the Institute

Diabetic retinopathy is a leading cause of blindness. The development of potent ARIs is of clinical significance in preventing blindness associated diabetes. Animal studies have demonstrated that ARIs prevent the selective loss of pericytes, the initial pathology of retinopathy. The observation that the polyol pathway is also present in fibroblast suggests that excess amounts of sugar alcohol may also play a role in the formation of proliferative tissues in advanced stages of retinopathy. This evidence provides additional rationales for the use of ARIs in the prevention and intervention of diabetic retinopathy.

Proposed Course

The evaluation of the polyol pathway will be continued with various tissues where diabetic changes occur. Cell culture techniques will be used to investigate retinal pericytes and endothelial cells as key cells in diabetic retinopathy.

NEI Research Program

Retinal Diseases—Diabetic Retinopathy, Sickle Cell Retinopathy and Other Vascular Abnormalities

Publications

Obrosova I, Inoue J, Greentree W, Sato S, Rodrigues L, Kador PF: Evaluation of S-88-0773 on sugar cataract formation. *Invest Ophthalmol Vis Sci* 24(suppl):1931, 1994.

Sato S, Kador PF: Diabetic retinopathy. I. Retinal changes in animal models. *Diabetes Frontier* 5:108-112, 1994.

Sato S, Old S, Carper D, Kador PF: Purification and characterization of recombinant placental and rat lens aldose reductases expressed in *Escherichia coli*. *Adv Exp Med Biol,* in press.

Schaffhauser MA, Sato S, Kador PF: NADPH-dependent reductases in dog thyroid: Comparison of a third enzyme "glyceraldehyde reductase" to dog thyroid aldehyde reductase. *Adv Exp Med Biol,* in press.

Secchi EF, Lisak MJ, Sato S, Kador PF: Presence of polyol pathway in fibroblast. *Invest Ophthamol Vis Sci* 34(4 suppl):1589, 1994.

Laboratory of Retinal Cell and Molecular Biology

Report of the Chief
Laboratory of Retinal Cell and Molecular Biology

Gerald J. Chader, Ph.D.

Members of the Laboratory of Retinal Cell and Molecular Biology (LRCMB) elucidate new genes and biochemical mechanisms to better understand the underlying causes of ocular diseases. With this knowledge, we hope to intervene better in the disease process before substantial damage to vision has been done or to apply rational methods of gene therapy before the terminal stages of the disease have been reached. The approaches taken are molecular biological, molecular genetic, and studies on candidate genes. The following three areas are emphasized:

- Molecular Biology and Molecular Genetics
- Gene Therapy of Retinal Diseases
- Molecular Immunopathology

Following are specific advances within these areas:

Molecular Biology and Molecular Genetics

(1) Retina-Specific Genes. Several genes that are predominantly or exclusively expressed in ocular tissues have been identified by subtractive cloning. These include an important gene HIOMT that is involved in the maintenance of circadian rhythms within the eye. Retina-specific genes and genes located on the short arm of the X chromosome have been pinpointed. These genes are being chromosomally localized to see if they are linked to eye diseases. Genomic cloning and sequencing are being done such that appropriate restriction fragment length polymorphisms and/or microsatellite repeats are generated to allow for disease testing.

(2) Retinal pigment epithelium (RPE)-Specific Genes. Cloning of genes unique to RPE and its functioning is of importance because the RPE cell layer is critical for retinal homeostasis. A new 65 kDa protein of potential importance has been isolated from the human RPE. The bovine and mouse genes have been cloned, allowing for study of tissue-specific expression. The gene is highly conserved, and its posttranslational expression is tightly regulated. This gene is the first RPE-specific gene to be reported and characterized. Knowledge of the 5′ regulatory sequence should facilitate RPE-specific gene transfer and gene therapy.

(3) Interphotoreceptor Retinoid-Binding Protein (IRBP). IRBP is an integral part of the visual cycle. Collaborative studies with Dr. Harris Ripps, from the University of Illinois at Chicago, on a model of experimental retinal detachment have demonstrated that IRBP is intimately involved in rhodopsin regeneration. We also have identified a protein homologue and cloned the homologous gene of human IRBP from the fruitfly *Drosophila melanogaster*. The gene maps to an area of the *Drosophila* genome that is rich in mutants of ocular disease. We hope to pinpoint a specific human population with similar characteristics and examine the gene for defects in specific human families.

(4) Pigment Epithelium-Derived Factor (PEDF). PEDF is synthesized by human RPE cells and may be important in the development of retinal photoreceptors. PEDF induces the extension of elaborate neuronal processes from cultured retinoblastoma cells and, as such, is a neurotrophic protein. Because Y-79 cells are thought to be derived from photoreceptor cone cells, it is hoped that PEDF can be as effective on cone neuron development *in vivo*. Collaborative studies have also shown PEDF to be a neuron-survival factor. The clinical use of PEDF in retinal transplantation is thus a distinct possibility. The molecular biology of this potentially very important neurotrophic and neuron-survival protein is being studied for application to retinal degenerations.

(5) Fatty Acid and Tubulin Defects in Retinal Degeneration. In collaborations with Dr. Muriel I. Kaiser, fatty acid uptake and metabolism in Bietti's crystalline retinopathy and a tubulin acetylation defect in a form of atypical retinitis pigmentosa are being investigated in hopes of elucidating the specific defects. Significant progress has been made in pinpointing the metabolic problems expressed in both these hereditary conditions.

Gene Therapy of Retinal Diseases

(1) Transgenic Studies. Transgenic studies can help to uncover factors controlling gene activation in the embryonic period, specifically in retinal photoreceptor cells. Gene analysis systems in transgenic mice and in transient transfections in cultured human retinoblastoma cells have been established for IRBP. Much of the 5'-flanking region of IRBP has been determined, and enhancer elements necessary for expression are being defined through target mutagenesis studies. Tissue- and stage-specific elements, including TATA and CAAT boxes, are being exactly defined as to retinal expression. This work is important so that specific molecules can be "gene-targeted" to the retina with precision.

(2) Gene Therapy. Ribozymes are specifically constructed ribonucleic acid species that can control expression of proteins within cells. By linking these simplified gene forms to appropriate promoters and using a suitable transfer vector, new therapeutic modalities can be constructed. Gene therapy can then be planned to treat autosomal dominant disorders that are unmanageable. Ribozyme constructs for IRBP have been designed and are being studied in a transfected human retinoblastoma cell system. Once perfected, ribozymes should be useful in conditions such as diabetic retinopathy and retinopathy of prematurity, in which the disorders probably involve overexpression of normal proteins such as growth factors.

Molecular Immunopathology

(1) Immunopathology. Work with Dr. Igal Gery continues to study aspects of the IRBP-induced uveitis seen in models of human uveoretinitis. Studies in the human are also under way, with the final goal of controlling or preventing at least some forms of uveitis in man.

(2) Immunogenetics. With Dr. Rachel Caspi, an IRBP-mouse model for experimental autoimmune uveitis has been established that is very useful for studying the genetics of the disease and its relapsing characteristics.

(3) Antigen Presentation. Collaborative work with Drs. Mark de Smet and Robert Nussenblatt has previously demonstrated the presence of a cell-surface protein of B cells that specifically binds the major immunopathological determinant of IRBP. It is thought that this protein may function as a molecular chaperone in antigen presentation. Recently, three intracellular proteins from human B cells have been uncovered that bind specifically to the immunodominant, uveopathological epitope of the IRBP molecule. Two of these proteins are now known to be novel heat shock proteins. Elevation of serum antibody levels to HSP 70 was found to occur during

ocular inflammatory episodes in patients with Beçhet's disease. These studies could lead to better diagnosis and treatment of forms of ocular uveitis and serve as a focus for gene therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00070-17 LRCMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Vitamin A and Ocular Tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Barbara Wiggert	Ph.D.	Head, Section on Biochemistry	LRCMB, NEI
Others:	Kalpana Rengarajan	Ph.D.	Visiting Fellow	LRCMB, NEI
	R. Krishnan Kutty	Ph.D.	Senior Staff Fellow	LRCMB, NEI
	Todd Duncan	M.S.	Biologist	LRCMB, NEI
	Geetha Kutty	M.S.	Visiting Associate	LRCMB, NEI

COOPERATING UNITS *(if any)*

U. Lund, Sweden (T. van Veen, Ph.D.); U. Illinois Coll. of Med., Chicago (D. Pepperberg, Ph.D., T.-I. Okajima, Ph.D., H. Ripps, Ph.D.); Med. U.S.C. (R. Crouch, Ph.D., S. Hazard, Ph.D.); SLU Inst. F. Kir, Sweden (K. Narfstrom, D.V.M., Ph.D.); U. Hosp., Utrecht, The Netherlands (B. Zonnenberg, M.D., Ph.D.); Medical College of Georgia (S. Smith, Ph.D.); Emory Eye Center (J. Nickerson, Ph.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Biochemistry

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In the toad retinal pigment epithelium (RPE) eyecup it was demonstrated that interphotoreceptor retinoid-binding protein (IRBP) promotes the formation, as well as the release, of 11-*cis* retinal.

In the experimentally detached skate retina system, the introduction of ligand-free IRBP purified from bovine retina to the subretinal space significantly increased the rate of rhodopsin regeneration and more than doubled the amount of rhodopsin reformed in the darkness as compared with controls in which IRBP content of the subretinal space was diluted as a consequence of detachment and no purified IRBP was added back.

In the vitiligo mouse model of retinal degeneration, elevated retinoid levels were found in both the RPE and the liver. These results represent the first evidence of biochemical malfunction in this mutant and suggest that in the eye, the RPE is the primary site of the defect that leads to retinal degeneration.

A ~70 kDa glycoprotein binding both retinoids and fatty acids was purified to homogeneity from *Drosophila melanogaster* heads.

A method was developed, using reverse transcriptase-polymerase chain reaction (RT-PCR), for the quantitation of IRBP message in small amounts of tissue such as a single mouse pineal gland.

Three intracellular human B-cell proteins (~40, 72, and 74 kDa) that bind specifically to peptide 1169-1191, an immunodominant, uveitopathogenic determinant of bovine IRBP, were partially characterized by immunoblotting and microsequencing. The 40 kDa protein was identified as actin, and the 72 and 74 kDa proteins were determined to be members of the hsp 70 family. The 72 kDa protein had 40 percent homology with human hsp 72 from lung fibroblasts, and the 74 kDa protein had a high degree of homology with human hsp 78 glucose-regulated protein from liver. Elevation of serum antibody levels to hsp 70 were found to occur during ocular inflammatory episodes in patients with Behçet's disease.

Project Description

Additional Personnel

Igal Gery	Ph.D.	Head, Section on Experimental Immunology, LI, NEI
Rachel Caspi	Ph.D.	Visiting Associate LI, NEI
Tatiana Putilina	Ph.D.	Visiting Associate LRCMB, NEI
Mark de Smet	M.D.	Visiting Scientist LI, NEI

Objectives

The purpose of this research project is to investigate the role of specific retinoid-binding proteins such as interphotoreceptor retinoid-binding protein (IRBP) in mediating the action of retinoids in both normal and diseased ocular tissues.

Methods

Affinity chromatography, fluorescence spectroscopy, high-performance liquid chromatography (HPLC), SDS-polyacrylamide gel electrophoresis, Western blotting, Northern blotting, reverse transcriptase-polymerase chain reaction (RT/PCR), slot-blotting, and the enzyme-linked immunosorbent assay were used to study retinoid-binding proteins.

Major Findings

The formation of 11-*cis* retinal in the retinal pigment epithelium (RPE) and its release to extracellular medium containing IRBP were studied in the RPE-eyecup of the toad. The results not only indicate that IRBP promotes the formation (from all-*trans* precursor) as well as the release of 11-*cis* retinal but also suggest the preferred use of recently incorporated and esterified all-*trans* retinol in the 11-*cis* retinal synthesis in a "last in/first out" manner.

It has been shown that, compared with a normal eyecup preparation, the amount of rhodopsin regenerated and the rate at which it was resynthesized after bleaching were reduced about 50 percent when the skate retina was detached from its RPE and replaced immediately on the apical surface of the RPE.

In current studies, the detachment procedure was performed under fluid to dilute the IRBP content of the interphotoreceptor matrix. Fundus reflectrometry showed that allowing fluid to enter the subretinal space exposed by the detachment procedure caused profound deficits in both the rate and amount of rhodopsin that regenerated after bleaching. The introduction of ligand-free IRBP purified from bovine retina to the subretinal space significantly increased the rate of rhodopsin regeneration and more than doubled the amount of rhodopsin reformed in darkness. It appears that one important consequence of retinal detachment is the dilution of IRBP in the subretinal space, so that its replacement, particularly in cases of extensive rhegmatogenous detachments, may be beneficial for the recovery of retinal function in human patients.

The vitiligo mouse (C57BL/6-mi^{vit}/mi^{vit}) model of retinal degeneration exhibits a slowly progressing loss of photoreceptor cell nuclei, gradual loss of rhodopsin, and unevenly pigmented RPE. Analyses of retinoid levels and distribution between the neural retina and RPE in these mutant mice showed that retinyl palmitate levels were significantly higher in the mutant RPE than in control mice. Furthermore, all-*trans* retinol was elevated approximately fourfold above controls in the RPE of vitiligo mice. To assess possible systemic involvement in vitiligo mice, retinoids were evaluated in liver and plasma. Plasma retinol levels were normal, but mean liver total vitamin A levels in affected mice were approximately 1.7 times greater than controls. Analysis of esterified and unesterified retinoids in liver showed that retinyl palmitate levels were elevated. These results showing elevated retinoid levels in both the RPE and liver of the vitiligo mouse represent the first evidence of biochemical malfunction in this mutant and suggest that, in the eye, the RPE is the primary site of the defect that leads to photoreceptor cell degeneration. This study provides the

first evidence of altered systemic retinoid metabolism in vitiligo mice that is occurring, significantly, under normal dietary conditions.

A glycoprotein binding both retinoids and fatty acids has been purified to homogeneity from *Drosophila melanogaster* heads. This protein, which has an apparent molecular mass of ~70kDa on SDS-PAGE, is similar to the 140 kDa IRBP in that both proteins are glycosylated, both exhibit endogenous covalent and noncovalent fatty acid binding, and both exhibit similar binding affinities for 16-[9-anthryloxy] palmitic acid. The *Drosophila* protein has a higher affinity for retinol than that of IRBP.

A method for the radioanalytic estimation of amplification products generated by reverse transcription coupled to the polymerase chain reaction of the IRBP message in mouse retina and pineal using [α-^{33}P] was developed. This method allows the detection of the IRBP message in small amounts of tissue such as a single mouse pineal gland. Quantitation of the IRBP message using the G3PDH message as an internal standard was demonstrated.

Peptide 1169-1191 is an immunodominant, uveitopathogenic determinant of bovine IRBP that causes severe ocular inflammatory disease in the Lewis rat. Three intracellular-binding proteins, with apparent molecular masses of 72 and 74 kDa, from Epstein-Barr virus-transformed human B cells (from both normal subjects and Behçets patients) and stimulated with lipopolysaccharide bind specifically to this peptide. These binding proteins could be released from the peptide with adenosine triphosphate (ATP), showing that all three contain an ATP-binding site.

Partial characterization of these proteins by immunoblotting and microsequencing of peptides obtained by *in situ* digestion of specific protein bands demonstrated a 40 kDa protein to be actin and the 72 and 74 kDa proteins to be members of the heat shock protein (HSP) 70 family. The 74 kDa protein has a high degree of homology with human HSP 78 glucose-regulated protein from human liver, whereas the 72 kDa protein has 40 percent homology with a human HSP 72 from human lung fibroblasts and probably represents a new member of the HSP 70 family. It was also shown in a corollary study that elevation of serum antibody levels to HSP 70 occurred during ocular inflammatory episodes in Behçets patients. Large-scale purification of IRBP was continued for studies on the production of experimental autoimmune uveitis (EAU) in rats and mice and possible modes of suppression of the disease.

Significance to Biomedical Research and the Program of the Institute

Because of its importance in normal photoreceptor cell physiology, *i.e.*, in facilitating the transport of retinoids during the visual cycle as well as transport of fatty acids that are essential to normal function, abnormalities in IRBP function resulting from changes in concentration, distribution, or affinity for retinoids or fatty acids could be important either directly or indirectly in visual cell pathogenesis.

Proposed Course

The physiological role of IRBP in the visual cycle, in particular, the mechanism by which it promotes the formation and release of 11-*cis* retinal, will continue to be probed. We will also be seeking to identify the epitopes on the IRBP molecule that bind retinoids and fatty acids as well as those that are required for eliciting the release of 11-*cis* retinal from the RPE. Continued studies on the vitiligo mouse model of retinal degeneration will assess retinoid turnover in liver and RPE as well as the consequences of manipulation of dietary vitamin A on levels of IRBP and retinoids and on photoreceptor degeneration in the eye. The *Drosophila* head retinoid and fatty acid-binding protein will be further characterized by cloning and sequencing and compared with IRBP.

The 72 kDa protein, which binds the uveitogenic peptide 1169-1191 of IRBP and appears to be a new member of the HSP 70 family of

HSPs will be investigated further by cloning and sequencing. Antibodies to this protein will also be obtained to study its possible role in antigen presentation. We will continue to conduct large-scale purification of IRBP protein for studies of EAU.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Caspi RR, Chan C-C, Grubbs BG, Silver PB, Wiggert B, Parsa CF, Bahmanyar S, Billiau A, Heremans H: Endogenous systemic interferon-gamma has a protective role against ocular autoimmunity in mice. *J Immunol* 152:890-899, 1994.

Duffy M, Sun Y, Wiggert B, Duncan T, Chader GJ, Ripps H: Interphotoreceptor retinoid binding protein (IRBP) enhances rhodopsin regeneration in the experimentally detached retina. *Exp Eye Res* 57:771-782, 1993.

Duncan T, Kutty G, Chader GJ, Wiggert B: A glycoprotein binding retinoids and fatty acids is present in *Drosophila*. *Arch Biochem Biophys* 312:158-166, 1994.

Kutty RK, Kutty G, Duncan T, Nickerson J, Chader GJ, Wiggert B: Radioanalytic estimation of amplification products generated by reverse transcription PCR using [α-^{33}P] deoxyribonucleoside triphosphate. *Biotechniques* 15:808-812, 1993.

Kutty RK, Nagineni CN, Kutty G, Hooks JJ, Chader GJ, Wiggert B: Increased expression of heme oxygenase-1 in human retinal pigment epithelial cells by transforming growth factor-β. *J Cell Physiol* 159:371-378, 1994.

Kutty RK, Kutty G, Rodriguez IR, Chader GJ, Wiggert B: Chromosomal localization of the human heme oxygenase genes: Heme oxygenase-1 (HMOX1) maps to chromosome 22q12 and heme oxygenase-2 (HMOX2) maps to chromosome 16p13.3. *Genomics* 20:513-516, 1994.

Kutty G, Duncan T, Nickerson JM, Si JS, van Veen T, Chader GJ, Wiggert B: Light deprivation profoundly affects gene expression of interphotoreceptor retinoid-binding protein in the mouse eye. *Exp Eye Res* 58:65-75, 1994.

Kutty RK, Kutty G, Nagineni CN, Hooks JJ, Chader GJ, Wiggert B: RT-PCR assay for heme oxygenase-1 and heme oxygenase-2: A sensitive method to estimate cellular oxidative damage. *Ann N Y Acad Sci*, in press.

Okajima TL, Wiggert B, Chader GJ, Pepperberg DR: Retinoid processing in the retinal pigment epithelium of the toad (Bufo Marinus). *J Biol Chem* 269:21983-21989, 1994.

Pepperberg DR, Okajima TL, Wiggert B, Ripps H, Crouch RK, Chader GJ: Interphotoreceptor retinoid-binding protein (IRBP). Molecular-biology and physiological role in the visual cycle of rhodopsin. *Mol Neurobiol* 7:61-85, 1993.

Rajagopalan S, Rodrigues MM, Wiggert B, Advani SH, Nair CN, Nickerson JM: Retinoblastoma: Interphotoreceptor retinoid-binding protein mRNA analysis by polymerase chain reaction. *Ophthalmic Paediatr Genet* 14:117-125, 1993.

Rengarajan K, de Smet MD, Chader GJ, Wiggert B: Identification of heat shock proteins binding to an immunodominant uveitopathogenic peptide of IRBP. *Curr Eye Res* 13:289-296, 1994.

Sasamoto Y, Kawano YI, Wiggert B, Chader GJ, Gery I: Induction of unresponsiveness in adult rats by immunodominant and nondominant peptides. *Cell Immunol* 152:286-292, 1993.

Smith MA, Kutty RK, Richey PL, Chader GJ, Wiggert B, Petersen RB, Perry G: Heme oxygenase-1 is associated with the neurofibrillary pathology of Alzheimer Disease. *Am J Pathol* 145:42-47, 1994.

Smith SB, Duncan T, Kutty G, Kutty RK, Wiggert B: Elevation of retinyl palmitate in eyes and livers and of IRBP in eyes of vitiligo mutant mice. *Biochem J* 300:63-68, 1994.

Wiggert B, van Veen T, Kutty G, Lee L, Nickerson J, Si JS, Nilsson EG, Chader GJ, Narfstrom K: An early decrease in interphotoreceptor retinoid-binding protein gene expression in Abyssinian cats homozygous for hereditary rod-cone degeneration. *Cell Tissue Res,* in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00297-01 LRCMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Microtubule Stability as a Factor in Retinal Degenerations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Susan Gentleman Ph.D. LRCMB, NEI

COOPERATING UNITS *(if any)*

Genetics & IVF Institute, Fairfax, VA (R. Sherins, M.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Gene Regulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [x] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Instability of the microtubules of the connecting cilia has been suggested as a cause or complicating factor in some cases of retinitis pigmentosa. These microtubules are normally highly acetylated on Lys40 of α tubulin, a posttranslational modification that appears to be restricted to polymerized microtubules; the function of this acetylation is as yet unknown. We have described the case of an infertile male subject with a rod-dominant retinal degeneration whose sperm flagella show severe morphological abnormalities and greatly reduced acetylation. Currently, the project is directed toward characterization of tubulin acetyl transferase (TAT) using bovine retina and brain as the source. TAT has been purified about 1,000-fold and is in a complex of about 300 kDa. SDS-PAGE analysis of the complex shows four major and several minor bands ranging from 30 to 120 kDa. The major bands are N-terminal blocked and will require digestion and purification of peptides before sequencing can be done. Arrestin (S-antigen) copurifies with the TAT complex from retina and is a substrate for the acetyltransferase activity. A 70 kDa band of τ-immunoreactive material is also found in the TAT complex from both brain and retina. The partially purified TAT complex from Y-79 retinoblastoma cells is under evaluation for use in future studies of the molecular biology of the TAT complex.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Muriel I. Kaiser	M.D.	Chief, OGCSB, NEI
W. Gerald Robison	Ph.D.	Chief, Section on Pathophysiology, LMOD, NEI

Objectives

Characterization of the components of the microtubule-associated acetyl transferase activity will be used to obtain molecular probes for analysis of deoxyribonucleic acid (DNA) from human subjects with retinal degenerations in which microtubule instability is suspected.

Methods

An assay of acetyl transferase activity in crude tissue fractions has been developed. An acetyl coenzyme A agarose affinity column, with attachment through the phosphate using phosphoramidate chemistry, has been made. Conventional techniques of protein chemistry and purification are used.

Major Findings

(1) Sperm flagella from an infertile male subject with a rod-dominant retinal degeneration were evaluated for alpha tubulin isoforms. The level of tubulin acetylation was 30 percent of the normal population, although tyrosinated tubulin level was normal. The tubulin acetyl transferase-specific activity of these flagella was also significantly lower than normal.

(2) The acetyl transferase activity from bovine brain and retina has been purified about a thousandfold from the subcellular fraction containing cold-stable microtubules (27,000 xg pellet) by high-salt extraction, anion-exchange chromatography, gel filtration by high-performance liquid chromatography, and affinity chromatography on AcCoA-agarose. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the purified fractions shows four major bands and several minor bands from both brain and retina. The major bands from brain have been eluted from gels for N-terminal analysis. These bands appear to be homogeneous but all are N-terminally blocked and cannot be sequenced without further processing.

(3) One band in the retinal complex is acetylated *in vitro* by incubation of the purified complex with AcCoA. This protein has been identified as arrestin (S-antigen) both by N-terminal sequencing and by immunoblotting.

(4) The purified complexes from both brain and retina were analyzed by immunoblotting with a panel of monoclonal antibodies against a variety of microtubule binding proteins (MAPs). A 70 kDa band of τ immunoreactivity was found in both preparations. Treatment of the complex with alkaline phosphatase appeared to increase the immunoreactivity of the 70 kDa band to the antibody τ-1, which specifically recognizes the unphosphorylated form of τ.

(5) The acetyl transferase complex from Y-79 retinoblastoma cells was partially purified and characterized by immunoblotting. Arrestin immunoreactivity copurified with the complex. The 70 kDa band of τ immunoreactivity in the complex from these cells also reacted with the MAP2 monoclonal antibody HM-2 and did not show an increase in τ-like immunoreactivity with dephosphorylation.

Significance to Biomedical Research and the Program of the Institute

The microtubule acetyl transferase complex is a multifunctional complex containing the enzyme with apparently a broad specificity and microtubule-binding proteins as well as other proteins, some of which are tissue specific. The single band of τ immunoreactivity suggests that a particular isoform is used by this complex. Therefore, a genetic defect in one splice site of τ might have major effects on the function of the complex. However, the

data from Y-79 cells indicate that other MAPs may substitute for the τ-like form, although possibly not as well. The association of arrestin with the complex in retina also suggests that it may play a role in the trafficking of arrestin in photoreceptor cells. Therefore, the retina might be particularly sensitive to malfunction of the complex.

Proposed Course

The current direction of the project is to continue the characterization of the components of the acetyl transferase complex. With this information, we intend to obtain molecular probes suitable for screening human material.

(1) Protein sequence of components of the complex will be obtained by digestion and purification of peptides from the proteins eluted from SDS-PAGE. The N-terminal blockade in most of the proteins of the complex requires that initial protein sequence data be obtained from internal peptides. Sequences will be matched to those in the available protein databases (*e.g.*, Swiss Prot) for identification.

(2) Peptide sequences not matching sequence in current data banks will be used to design degenerate oligos for polymerase chain reaction probing of complementary cDNA libraries with the objective of obtaining inferred protein sequence for the complete proteins.

(3) From the sequence information obtained, molecular probes will be made to examine human genes. Family pedigrees of type 2 Usher's syndrome are of particular interest. Several reports from various laboratories have demonstrated axonemal abnormalities at a higher than normal frequency in some of these people.

NEI Research Program

Retinal Diseases—Photoreceptors and Pigment Epithelium

Publications

Lloyd RA, Gentleman S, Chader GJ: Assay of tubulin acetyl transferase activity in subcellular tissue fractions. *Anal Biochem* 216:42-46, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00196-11 LRCMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Genetics of the Eye and Ocular Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Diane E. Borst	Ph.D.	Senior Staff Fellow	LRCMB, NEI
	Steven Bernstein	Ph.D., M.D.	Senior Staff Fellow	LRCMB, NEI

COOPERATING UNITS *(if any)*

University of Texas, Dallas (R. Hammer, Ph.D.); University of Illinois (H. Ripps, Ph.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Gene Regulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Antisense refers to a nucleic acid molecule that is complementary to an expressed messenger RNA sequence present in an organism. We are evaluating the use of various forms of antisense molecules, both catalytic (ribozymes) and noncatalytic in nature, to evaluate the function of specific gene expression in the eye. Ribozymes complementary to interphotoreceptor retinoid-binding protein mRNA have been evaluated *in vitro* and *in vivo*. Antisense to aldose reductase is currently being evaluated *in vivo*.

Project Description

Additional Personnel

Eric Wawrousek	Ph.D.	Research Biologist, OSD, NEI
Susan DiCamillo	B.S.	Chemist, OSD, NEI

Objectives

Antisense and catalytic antisense are being used to downregulate specific functions in ocular tissues for the purposes of both basic research and clinical application.

This research is designed to define the *cis*-acting elements in the mouse interphotoreceptor retinoid-binding protein (IRBP) promoter that controls the regulation of the IRBP gene expression. Other factors such as deoxyribonucleic acid (DNA) methylation may be involved in the regulation of IRBP gene expression; another objective is to define the DNA methylation state of the IRBP promoter in different tissues.

Methods

This study uses *in vitro* transcription and assay methodology as well as transgenic expression of antisense constructs in mice and rats. Conventional techniques for cloning and analysis of nucleic acids are used.

Transgenic mice are being used to study the *cis*-acting elements of the IRBP promoter. The transgene contains varying lengths of the mouse IRBP promoter fused to the reporter gene, chloramphenicol acetyl transferase (CAT). The levels of CAT activity in these mice show which DNA sequences are important for the tissue and developmentally specific expression of the IRBP gene. DNA was isolated from different tissues, digested with Msp I or Hpa II, Southern blotted, and probed with fragments of the IRBP promoter to study its DNA methylation state.

Major Findings

Most attempts at using ribozymes for downregulation and "gene therapy" in human immunodeficiency virus have used ribozymes of varying complement length; this complement length gives specificity to the target site of the message. The optimal length for this enzyme-target interaction is unknown, and this is partially confirmed by the high failure rate and exceedingly variable activity rates for engineered molecules against various targets. Using *in vitro* partial duplex transcription, cloned ribozyme templates, and substrate fragments, I have studied the effect of site specificity and varying complement length on ribozyme activity *in vitro*. I have found that ribozyme activity can be "tuned" *in vitro* by varying complement length and that this tuning is unique and target-site specific. The current computer-based programs for predicting site sensitivity are inadequate to this task; it must be performed empirically. This study is completed.

With the help of the above technique, I have generated two constructs containing ribozymes targeted against different sites in the messenger ribonucleic acid (mRNA) for IRBP, which have the highest demonstrated *in vitro* activity and have generated transgenic founder lines expressing these ribozymes in ocular and other tissues. The data from this study is preliminary, but there are apparently significant differences in the embryonic survival and tissue-specific expression of transgene and IRBP mRNA in the different constructs. We are currently doing ocular histology studies on outbred animals (F1 generation).

With the collaboration of Dr. Harris Ripps, from the University of Illinois at Chicago, we have performed electrophysiological studies on these mouse lines. There are apparent differences in some of the transgenic lines in electroretinogram responses, and these apparently correlate with transgene expression. Animal lines apparently expressing the highest levels of the transgene also demonstrate high embryonic fatality. Thus, IRBP expres-

sion may be important in fetal development. This work correlates well with earlier reports of IRBP expression in fetal tissue of both rats and cows.

Aldose reductase (AR) is believed to play a key role in the development of diabetic retinopathy, neuropathy, and nephropathy. Additionally, AR is apparently involved in cataractogenesis in diabetic humans and galactose-fed rats. Research to date has focused on AR inhibitors in animal systems, these inhibitors must be administered chronically and are expensive; in addition, in one of the most widely studied animal systems, the dog, development of retinopathy requires an extended period of time. Studies in mice are hampered by the fact that they do not develop histologically documented retinopathy or neuropathy, although they apparently exhibit nerve conduction velocity changes.

Rats also develop diabetic changes and are an alternative, relatively low-cost mammalian system in which to study progression of diabetic pathology. Rat transgenic model systems are now available. Because the mRNA sequence of rat AR is known, we have designed gene constructs containing antisense-based sequence to rat AR. Expressed *in vivo,* AR deficient rats should show delayed galactose-induced cataractogenesis and delayed onset of diabetic histopathology. In collaboration with Dr. Robert Hammer, from the Howard Hughes Medical Research Institute in Houston, Texas, I am generating strains of rats that express antisense for AR in a variety of tissues. This work is in the animal production stage.

Tissue culture correlates of retinoblastoma. We have characterized two mouse retinoblastoma cell lines in terms of photoreceptor-specific gene expression. These lines may be useful in the future for *in vitro* transfection studies using antisense technology without the need for transgenic mice production. This study is completed.

Retinopathy of prematurity (ROP) is one of the leading causes of early childhood blindness in the United States. Pathology in this disease results from the development of retinal neovascularization in premature or low birth weight infants that can then progress, in its most severe form, to invasion of the vitreous body, and either during the rapid period of vessel growth or following involution, to retinal detachment. The only effective treatment is surgical, with 25 to 35 percent of the most severely affected infants maintaining vision of 5/200 or less.

Dr. Lois Smith, from the Massachusetts Eye and Ear Infirmary, Boston, Massachusetts, has developed a reproducible model of ROP in newborn mice. In collaboration with Dr. Smith, I have prepared a series of antisense molecules that I am testing for efficacy of specific inhibition of mitosis. These compounds will then be used to determine efficacy of inhibition of ROP *in vivo.*

Msp I and Hpa II are isoschizomers that are methylation dependent. Hpa II will not digest the recognition sequence if the 3' cytosine is methylated. Msp I sites of the IRBP 5' flanking region were studied: five in the cow and two in the mouse. One of these sites is in the homologous location and is hypomethylated only in the retina. Similar results have been found for the other Msp I sites that have been studied. Perhaps these regions of the IRBP promoter are important in the regulation of IRBP expression either by being a direct protein-binding site or by somehow influencing the secondary structure of this region.

Significance to Biomedical Research and the Program of the Institute

In vitro testing of ribozyme activity may enable the selection of unique ribozymes, with the possibility of enhanced *in vivo* action, for use in both gene therapy and basic research. The transgenic animal results may tell us much about the role of IRBP in embryonic development as well as the function of the retina during relative IRBP deficiency. Generation of transgenic rats expressing antisense for AR may be useful in evaluating the direct

pathophysiological link between AR activity and pathology in diabetes. Evaluation of antisense molecules with antimitotic activity will likely be useful in direct pharmacological treatment of retinopathy of prematurity.

IRBP in adults is expressed only in the retina and pineal gland. Understanding the mechanisms and the nucleotide elements that control IRBP expression is fundamental to understanding retinal development and function. This understanding could help us design drugs that would specifically target the retina and perhaps would also elucidate abnormal retinal function.

Proposed Course

Evaluation of mouse retinoblastoma cells: completed. *In vitro* evaluation of hammerhead ribozyme activity: completed.

Transgenic animal studies/expression of ribozyme activity. I am breeding transgenic animal lines expressing the active ribozyme constructs to select for those lines with the highest homozygous expression of ribozyme activity. I anticipate sending some of these adult animals to Dr. Ripps for further electrophysiological studies. In addition, I am attempting to determine the time of onset of lethality of the ribozyme expression in the prenatal animal.

AR antisense/transgenic rats. I am awaiting initial confirmation that Dr. Hammer has indeed generated a strain of rats expressing the AR antisense construct that I sent him. When this is completed, I will begin evaluation of *in vivo* expression of the construct. Initial feedings with high galactose will be performed to determine the speed of onset of cataractogenesis *vis-a-vis* control rat strains. Further analysis depends of the initial delivery on these animals.

Determination of antineovascular activity of antisense compounds. Initial studies determining the specific antimitotic activity are under way. The compounds possessing the highest antimitotic activity will be evaluated for their pharmacotherapeutic index *in vitro.* These specific compounds will be evaluated further for their relative antineovascular activity *in vivo.*

NEI Research Program

Retinal Diseases—Photoreceptors and Retinal Pigment Epithelium

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00124-14 LRCMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of the Retina and Pigment Epithelium

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gerald J. Chader	Ph.D.	Chief	LRCMB, NEI
Others:	R. Theodore Fletcher	M.S.	Chemist	LRCMB, NEI
	Joyce Tombran-Tink	Ph.D.	IRTA Fellow	LRCMB, NEI
	S. Patricia Becerra	Ph.D.	Visiting Scientist	LRCMB, NEI
	Timothy Schoen	M.S.	Biologist	LRCMB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Gene Regulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	2.5	1.5

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The title of this project has been changed to more accurately reflect the thrust of the science. The retina and pigment epithelium are neuroepithelial tissues that work in close cooperation. Specific growth and differentiating factors are found in the eye that guide development and interactions of individual ocular tissues to form a functional visual system. Studies on this project are focused on an understanding of the molecular biology and molecular genetics of the retina and discovering new genes that are candidates for hereditary retinal degenerations. For example, ocular tissues synthesize a number of growth factors. There now appear to be several systems present that could self-regulate growth and metabolic activity in the retina-pigment epithelium complex and be involved in eye diseases. In this regard, we have cloned and characterized a unique protein secreted from fetal human pigment epithelial cells, called pigment-epithelial derived factor (PEDF), that is "neurotrophic" to cultured human retinoblastoma cells and may affect neural retinal development *in vivo*. This protein also is a potent "neuronotrophic" agent in that it promotes neuronal cell survival of cultured cerebellar granule cells. Finally, PEDF is "gliastatic" in that it markedly retards glial cell growth. Along with being a candidate gene in retinal degenerations, the uses of PEDF in neuronal transplant in retina and other CNS areas are obvious.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Joan Schwartz	Ph.D.	Chief, Molecular Genetics Section, DIR, NINDS

Objectives

Our objective is to obtain a better understanding of the molecular biology and molecular genetics of ocular tissues in health and disease. The study of growth and differentiation factors, be they protein (*e.g.*, pigment epithelium-derived factor [PEDF]) or polypeptide (*e.g.*, insulin-like growth factor [IGF-1]) is critical in obtaining a view of the events that control the early development of the eye and also in maintaining normal function in the adult.

Methods

Molecular biological, genetic, and immunocytochemical techniques are used. Tissue culture is performed on cultured cells. In particular, the human retinoblastoma cell line Y-79 is used as a test system for differentiating agents.

Major Findings

Hereditary diseases are often caused by defects in genes that are important in cell division and differentiation; PEDF seems to be such a gene product. It is secreted from fetal pigment epithelial cells and is present in normal adult interphotoreceptor matrix. The protein migrates at approximately 50 kDa on sodium dodecyl-sulfate-polyacrylamide gels. Importantly, PEDF causes marked differentiation of human Y-79 retinoblastoma cells in culture (neurotrophic effect). This is characterized by an extensive elongation of neurite-like processes and a gathering of cells into "rosette-like" aggregates. Immunocytochemically, the expression of specific neuronal markers is also enhanced. Thus, PEDF is a unique protein, synthesized and secreted by retinal pigment epithelial cells, that could direct early development even in early embryogenesis. PEDF is also present after the important developmental period and may help to maintain retinal cell viability (neuron survival) in the adult retina. PEDF is also a candidate gene in the retinal dystrophy observed in the Royal College of Surgeons rat.

After the cloning of the complementary deoxyribonucleic acid for the PEDF gene, we have determined that the protein is a member of the SERPIN (serine protease inhibitor) superfamily of genes. One other member of this family is known to promote neuronal differentiation so it makes it more probable that PEDF has a major, and similar, role in the retina. The recombinant protein (rPEDF) has now been expressed in *Escherichia coli* cells and has been shown to be an active neurotrophic agent. The availability of relatively large amounts of PEDF should allow for more direct studies on its role(s) in ocular development and disease. We also find PEDF messenger ribonucleic acid in ciliary body and PEDF protein in vitreous humor, indicating that PEDF could be of importance in ocular tissues other than the retina.

In work with Dr. Joan Schwartz, National Institute of Neurological Disorders and Stroke (NINDS), we have evidence demonstrating that PEDF is also a potent "neuronotrophic agent," *i.e.*, neuron survival factor in a cultured cerebellar granule cell (CGC) system. Very small amounts of rPEDF added to the CGCs are effective in keeping the cells alive for prolonged periods of time. Surprisingly, we have found that PEDF has "gliastatic" properties as well, in that glial cells in the cerebellar cultures are markedly inhibited. Importantly, this is a long-term effect; a one-time exposure to nanogram quantities of rPEDF inhibits growth of cultured cerebellar glia for up to 12 weeks.

Significance to Biomedical Research and the Program of the Institute

Determining the genes that control normal ocular growth, differentiation, and function

and studying them on a molecular biological and molecular genetics level will aid us in understanding diseases of the eye, especially those of a hereditary, early developmental nature. With such knowledge, rational methods of gene therapy can be applied to ocular diseases. Because of its potent neurotrophic, neuronotrophic, and gliastatic effects, PEDF seems to be of potential use in retinal and central nervous system transplantations.

Proposed Course

The molecular biology and molecular genetics of ocular development will be further examined. The factors that affect normal and abnormal growth will be investigated. The full PEDF gene will be examined and analyzed to help elucidate its presumptive role(s) in retinal development. The rPEDF protein will be used to elucidate the role of the novel new protein in retinal diseases processes.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00148-21 LRCMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visual Control Mechanisms and Hereditary Degeneration

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gerald J. Chader	Ph.D.	Chief	LRCMB, NEI
Others:	Paul Wong	Ph.D.	Visiting Fellow	LRCMB, NEI
	Tatiana Putilina	Ph.D.	Visiting Associate	LRCMB, NEI
	Ignacio Rodriguez	Ph.D.	Staff Fellow	LRCMB, NEI
	June Lee	M.D.	Visiting Associate	LRCMB, NEI
	Timothy Schoen	M.S.	Biologist	LRCMB, NEI

COOPERATING UNITS *(if any)*

School of Veterinary Medicine, Cornell University (G. Aguirre, D.V.M., Ph.D.); Department of Zoology, University of Lund, Lund, Sweden (T. van Veen, Ph.D.); Instituto Nazionale per la Ricera sul Cancro, Genova, Italy (A. Albini, Ph.D., D. Noonan, Ph.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Gene Regulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	4.5	0.5

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The title of this project has been changed to more accurately reflect the thrust of the science. We are studying the expression of specific gene products that could be related to hereditary diseases of the retina. If normal genetic control mechanisms fail, hereditary diseases of the retina such as retinoblastoma or retinitis pigmentosa will result. We have now developed new techniques to clone and sequence retina-specific genes at a higher efficiency. We have found several genes that are either expressed exclusively or predominantly in the retina and are using these as candidate genes in specific blinding diseases. Among these are hydroxy-O-methyl transferase, an important gene on the X chromosome involved in circadian control in the retina and a new gene mapping to chromosome 11q close to four important genetic retinal diseases. Similarly, we are investigating the properties of known retina-specific genes such as the interphotoreceptor retinoid-binding protein (IRBP) and their involvement in retinal disease processes. Progress has also been made in identifying apoptosis as a primary and unifying mechanism for cell death in several hereditary retinal degenerations. All these factors and processes could lead to more efficient gene therapy of the diseased neural retina.

Project Description

Additional Personnel

Muriel I. Kaiser M.D. Chief, OGCSB, NEI

Objectives

Expression of genes in the retinal photoreceptor neuron in a normal manner is crucial to visual function in the adult. Thus, the factors that code for normal gene control and expression in human retina and in animal models of retinal degeneration are of primary interest. We also have started a major effort to develop new molecular biological techniques such that unique retinal and retinal pigment epithelium (RPE) genes can be identified, cloned, and sequenced to be used ultimately in screening human populations with inherited diseases of the visual system.

Methods

Standard molecular biological, biochemical, and neurochemical techniques are used. Histochemical techniques are used when necessary.

Major Findings

(1) We have developed new molecular biological techniques that allow for more efficient identification of highly expressed genes of the retina-PE complex. Each tissue of the body expresses a unique complement of genes that are transcribed and translated at a high level. In the retina and pigment epithelium, several very specific proteins are highly expressed such that photoreception and the visual process can take place. In a similar vein, it is often a genetic defect in these tissue-specific genes that results in a hereditary degeneration such as retinitis pigmentosa. We have developed and are using new methods for rapid polymerase chain reaction-based construction of specifically enriched libraries from very small retinal samples. This is especially important because tissue samples are limited in studying early development and rare pathology samples. A significant methodological advance we have made involves subtractive cloning on an immobilizing Dynabead base®. In this way, several new genes have been found that are being used as candidate genes for hereditary retinal degenerative diseases.

(2) In collaboration with National Eye Institute (NEI) clinicians, we are now applying these techniques to the study of the role of several proteins and genes in retinal disease. Among these are:

(a) Fatty acid-binding proteins that may be involved in normal and degenerating retinas such as in Bietti's crystalline dystrophy.

(b) A newly discovered gene on chromosome 11q that is a prime candidate gene in Bardet-Biedl's syndrome, Best's disease, familial exudative vitreoretinopathy, and one form of Usher's syndrome.

(c) The hydroxyindole-O-methyl transferase (HIOMT) gene that maps to the short arm of the X-chromosome and is a key enzyme in melatonin production in the retina.

(3) Finally, we are beginning to understand the mechanism by which photoreceptor cells die in a number of hereditary retinal diseases. This process is called "apoptosis" or programmed cell death. We have found induction of the marker gene TRPM-2 (clusterin) in all cases of retinal degenerations studied. It thus appears that programmed cell death may be a common mechanism by which many hereditary defects initiate photoreceptor cell death.

Significance to Biomedical Research and the Program of the Institute

One approach to studying a hereditary disease process in a tissue and its reversal through gene therapy is to first identify the normal complement of unique genes expressed in that tissue. This is also applicable in an early degenerative process, *e.g.*, retinitis pigmentosa,

and in other hereditary diseases such as Bietti's crystalline dystrophy or Best's disease in which the disease may be systemic, but the disease primarily affects the retina. In parallel, if one knows the common mechanism by which photoreceptor cells actually die in the various retinal degenerations, it may be possible to design strategies by which retinal cells are spared, at least long enough for transplantation or gene therapy experiments to be successful. Thus, studying apoptosis and similar processes in the retina will lead to better methods for gene therapy in the neural retina.

Proposed Course

We will continue to study molecular biological and developmental control mechanisms in the retina and pigment epithelium. In particular, we will investigate gene expression in normal retinas and in retinas affected with specific genetic diseases and focus on subtractive cloning as a prime method for identifying defective or missing genes in retinal diseases. Apoptosis will continue to be an important area for study because future gene therapy in retinal degenerations may depend on understanding how to prevent death of the photoreceptor neuron.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Diseases

Publications

Chader GJ: Retinal degenerations of hereditary, viral and autoimmune origins: Studies on opsin and IRBP, in Osborne N, Chader GJ (eds): *Progress in Retinal and Eye Research.* Oxford, Pergamon Press Ltd, 1994, vol 13, pp 65-99.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00260-05 LRCMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Outer Retina-Specific Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Michael Redmond	Ph.D.	Research Biologist	LRCMB, NEI
Others:	Suyan Liu	M.D., Ph.D.	Visiting Fellow	LRCMB, NEI

COOPERATING UNITS *(if any)*

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Laboratory of Retinal Cell and Molecular Biology

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Retinal pigment epithelium (RPE) cells and photoreceptor cells are functionally and developmentally closely integrated. Derangements of the RPE are involved in certain retinal diseases. However, the RPE is poorly understood at the molecular level. We have cloned and characterized RPE65, a novel developmentally-regulated conserved 65 kDa RPE-specific microsomal membrane-associated protein. The cDNA sequence is being used to overexpress RPE65 protein for functional studies. The potential role of the protein in inducing uveitis will also be studied using recombinant protein.

The RPE65 protein is not expressed in cultured RPE even though its mRNA is abundant. In characterizing this example of posttranscriptional regulation, we have identified distinct sequences in the 3′ untranslated region of the RPE65 mRNA that control the stability and the efficiency of translation of the RPE65 message. We have cloned and are sequencing a full-length human genomic clone for RPE65. It is at least 40 kilobases in length. Sequence analysis shows that the RPE65 protein is highly conserved between human and cow. We have also cloned the mouse gene. The human gene for RPE65 is localized to chromosome 1p31, and the mouse homolog to distal chromosome 3. These do not correspond to any ocular disease gene localized so far. Nonetheless, RPE65 remains a candidate gene for RPE-involved disease.

Project Description

Objectives

The retinal pigment epithelium (RPE) and the photoreceptor cell layer of the neural retina form a functionally and developmentally interdependent complex. Dysfunction of the RPE, accordingly, is deleterious to the photoreceptors and, hence, to vision itself. In spite of these important considerations, little is known about the RPE at the molecular level. In this laboratory, we are cloning proteins specifically or preferentially expressed in the RPE with a view to understanding mechanisms important to the RPE. At present, our major emphasis is on a 65 kDa protein that we have named RPE65. We are also studying other RPE-expressed proteins.

Methods

Molecular cloning and biochemical and protein chemistry techniques are used in this study. Additionally, we are using automated fluorescent deoxyribonucleic acid (DNA) sequencing and gene mapping techniques.

Major Findings

(1) RPE65 is a developmentally regulated, membrane-associated, nonglycosylated 65 kDa protein restricted to and conserved in vertebrate RPE and is the major protein of the RPE microsomal fraction. The protein displays an affinity for phospholipids that is calcium independent. We have cloned a composite 3,115 bp complementary DNA (cDNA) for this protein.

(2) We have found that distinct sequences in the 3′ untranslated region (UTR) of the RPE65 messenger ribonucleic acid (mRNA) regulate the stability of the RPE65 message and the efficiency of its translation. This is the first example of 3′ UTR-mediated regulation in an ocular gene.

(3) We have isolated a human genomic clone for RPE65. It is at least 40 kilobases in length. Much of the gene has been sequenced. Preliminary sequence analysis indicates that the RPE65 protein is highly conserved in evolutionary terms.

(4) We have localized the gene for the RPE65 to human chromosome 1p31 and to the far distal end of mouse chromosome 3. Neither of these loci matches that of a known ocular disease or phenotype. The mouse gene has been cloned to allow us to pursue transgenic and homologous recombination approaches for studying RPE65 gene regulation and function in the mouse.

Significance to Biomedical Research and the Program of the Institute

The RPE is poorly characterized at the molecular level. This is in spite of its pivotal role in the maintenance of photoreceptor function and, hence, of vision itself. We have identified RPE65 as a conserved RPE-specific molecule that is developmentally expressed. cDNA sequencing demonstrates that it is a novel protein. The function of this protein, although not yet clear, may be related to its affinity for phospholipids. Elucidation of the basis for its posttranscriptional regulation *in vitro* may have significant bearing on the culture of RPE cells. This is of some clinical significance because RPE cell transplantation is receiving much attention as a possible mode of intervention in treating some retinal diseases. In addition, because of its RPE specificity, the RPE65 gene can be considered a potential candidate gene for retinal disease.

At present, however, neither its human nor mouse chromosomal locations match those of any mapped disease loci. This may change as more disease loci are matched. Again, in view of its RPE-specific expression, elucidation of its gene structure may uncover RPE-specific regulatory elements. The high degree of conservation in the RPE65 protein suggests an evolutionarily important function for this protein. Finally, in view of the involvement of the RPE in uveitis, it is possible that RPE65 is uveitogenic. Because we have cloned the

cDNA, it will now be possible to overexpress the protein to test this hypothesis.

Proposed Course

(1) The translational efficiency regulating sequence in the 3' UTR will be characterized, as will the mRNA instability region. This will provide insights into translational regulation in the outer retina.

(2) Analysis of the structure of the human RPE65 gene will be continued. The remainder of the gene will be sequenced. Due to its importance as an RPE-specific gene, regulatory regions as well as the promoter will be identified and analyzed.

(3) The mouse RPE65 gene will be compared with the human RPE65 gene, especially with regard to the promoter region. Transgenic and homologous recombination "knockout" studies will be pursued to understand RPE65 function and regulation as well as to provide potential new models for human retinal disease.

(4) RPE65 will be tested as a possible RPE autoantigen. RPE65 protein will be overexpressed for this purpose.

(5) Elucidation of the structure and function of the RPE65 protein will continue. This will involve use of a variety of approaches. We will pay special attention to its possible role in retinoid and/or lipid trafficking.

NEI Research Program

Retinal Diseases—Photoreceptors and Pigment Epithelium

Publications

Hamel CP, Jenkins NA, Gilbert DJ, Copeland NJ, Redmond TM: The gene for the retinal pigment epithelium-specific protein RPE65 is localized to human 1p31 and distal mouse 3. *Genomics* 20:509-512, 1994.

Redmond TM, Jenkins NA, Gilbert DJ, Copeland NJ, Hamel CP: The gene for the retinal pigment epithelium-specific protein RPE65 is localized to human 1p31 and distal mouse 3. *Invest Ophthalmol Vis Sci 35(suppl)*:1312, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00250-07 LRCMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Experimental Autoimmune Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Toshimichi Shinohara	Ph.D.	Head, Section on Molecular Biology	LRCMB, NEI
Others:	Dhirendra Singh	Ph.D.	Visiting Associate	LRCMB, NEI
	Shirley Yu	B.S.	Biologist	LRCMB, NEI

COOPERATING UNITS *(if any)*

Department of Ophthalmology, Miami University, Miami, FL (D. Hamasaki, Ph.D.); Department of Anatomy, Nagoya University School of Medicine, Tsuramai, Showa-ku, Nagoya, Japan (Jiro Usukura, M.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously determined amino acid sequences of human, mouse, rat, and bovine retinal S-antigen and rat pineal gland S-antigen. Immunogenic sites and four uveitopathogenic sites of S-antigen were also determined. Two of the immunogenic sequences were highly conserved among these species.

Many proteins that have a similar sequence with a uveitopathogenic site are in the National Biomedical Research Foundation database. We chemically synthesized many peptides, and some of them induced experimental autoimmune uveitis (EAU) and experimental autoimmune pinealitis (EAP) in Lewis rats. In addition, native yeast histone H3 was also capable of inducing EAU.

To understand the role in autoimmunity of infectious micro-organisms that have cross-reactive antigens, we injected Lewis rats with peptide M together with one of six different killed bacteria, either with or without incomplete Freund's adjuvant (IFA). The rats injected with IFA developed EAU. To assess the impact of infection by live micro-organisms, low doses of live *E. coli* expressing S-antigen and baker's yeast, with a cross-reactive antigen, were injected several times into the rats. The rats injected with either live *E. coli* or live yeast developed EAU. We conclude that infection by micro-organisms that have cross-reactive antigens can break immune tolerance to self-antigens and induce inflammatory autoimmune diseases.

As an extension of our previous EAU research, we speculated that some types of cataracts may be induced by autoimmune insults. To investigate this issue we carried out similar experiments. Three groups of four rats were injected three times with lens homogenate, β-crystallins or a β-crystallin (β-A1) emulsified with complete Freund's adjuvant (CFA). All the animals developed severe damage in lens epithelial cells after 5 weeks from the date of the first injection. The rats injected with a synthetic peptide derived from *Salmonella typhimurium* protein that has five amino acid residues identical to rat β-crystallin (β-B2) also induced similar damage. Infection of microbes having homologous antigens to the lens antigens can induce the auto-antibodies at high levels that provoke damage to the lens epithelial cells. Thus, autoimmune insult in the lens epithelial cells may be an etiology of an initial stage of cataractogenesis. Direction of our future research will be to focus more on the autoimmunity in the lens cataractogenesis.

Project Description

Objectives

The objectives of this project are to understand the basic etiology of autoimmune inflammation, including uveitis, and to find possible treatments for human uveitis.

Methods

Conventional methods for analysis of proteins and nucleic acids are used. These include protein purification, ribonucleic acid (RNA) and deoxyribonucleic acid isolation, characterization and sequencing, molecular cloning, screening of clones, *in situ* hybridization, immunocytochemistry, and chromosome mapping. We also synthesized and used oligopeptides and oligonucleotides. Bovine, murine, primate, and human materials are used. Animal experiments were carried out with Lewis rats and monkeys. T-cell response and adaptive transfer were done with lymph node or spleen cells of rat.

Major Findings

(1) Local sequence homology was found between peptide M and several other foreign proteins, including potato proteinase inhibitor IIa, *Escherichia coli* hypothetical protein, Hepatitis B virus probable DNA polymerase, Moloney murine sarcoma virus gag-polyprotein, Moloney murine leukemia virus Gag-pol polyprotein, Baboon endogenous virus gag-pol polyprotein, and Baker's yeast histone H3.

(2) The synthetic peptides of the abovementioned proteins induced experimental autoimmune uveitis (EAU) in Lewis rats with similar pathology to EAU induced by peptide M or native S-antigen (S-Ag).

(3) For the first time, we proposed and showed the evidence that molecular mimicry plays a role in the process of pathogenesis of EAU and perhaps in autoimmune diseases in general.

(4) Oral administration of histone H3 peptide suppressed EAU in the Lewis rats.

(5) The suppression of EAU by histone H3 was also found in the EAU induced by the S-Ag. Thus, the tolerance also crossreacted between the peptide that has molecular mimicry.

(6) The T-lymphocytes obtained from rats immunized with peptide M or yeast histone H3 transferred disease, EAU, in the naive rats (adoptive transfer) when stimulated either with peptide M or histone H3. In addition, oral tolerance was also adoptively transferred from rats fed peptide M or histone H3 to the naive rats.

(7) Infection by microorganisms that have crossreactive antigens can break immune tolerance to a self-antigen and induce inflammatory autoimmune diseases.

Significance to Biomedical Research and the Program of the Institute

Uveitis is a leading cause of visual handicap in the United States and throughout the world. Some types of uveitis were suspected to be induced by bacterial and viral infections by many physicians for many decades. However, there is no clear link between infection and disease.

Autoimmune processes are thought to play a significant role in the pathogenesis of disease. Molecular mimicry, a process by which an immune response directed against a nonself protein crossreacts with a normal host protein, may play a role in autoimmunity. Here, we have proposed the idea of molecular mimicry and showed evidence that molecular mimicry may play a role in the pathogenesis of EAU. In addition, we have shown evidence that infection is a possible cause of autoimmune inflammation. These findings provide an important clue for understanding the etiology of autoimmune inflammatory diseases in humans.

Proposed Course

This project will be terminated.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Li Q, Abe T, Kikuchi T, Nussenblatt RB, Shinohara T, Chan C-C: Corticosteroids enhance S-antigen expression in non-retinal ocular tissue of rats with experimental autoimmune uveitis. *Exp Mol Pathol* 60:27-38, 1994.

Singh DP, Kikuchi T, Singh VK, Shinohara T: A single amino acid substitution in core residues of S-antigen peptide confers the capacity to prevent experimental autoimmune uveitis (EAU). *J Immunol* 152:4699-4705, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00132-13 LRCMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Phototransduction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Toshimichi Shinohara	Ph.D.	Head, Section on Molecular Biology	LRCMB, NEI
Others:	Takanobu Kikuchi	Ph.D.	Visiting Associate	LRCMB, NEI

COOPERATING UNITS *(if any)*

Mount Sinai Hospital, Toronto, Canada (Martin Breitman, Ph.D.); Department of Anatomy, Nagoya University School of Medicine, Tsurumai, Showa-Ku, Nagoya, Japan (J. Usukura, M.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have characterized the S-antigen genes from human and mouse, 33K protein genes from mouse and human. The S-antigen genes were approximately 50 kbp in length, contained 16 exons and 15 introns, and were composed of 97 percent intron and 3 percent exon. The 5′-flanking regions of the genes, approximately 1.5 kbp long, had no known regulatory elements for transcription such as TATA, GC, or CCAAT boxes.

Regulatory sequences and nuclear factors governing tissue-restricted expression of the mouse arrestin gene were investigated. The results showed that while proximal promoter sequence -38 to +304 are sufficient to direct low levels of retina-specific gene expression, sequences extending upstream to position -209 support higher levels of expression in the retina, as well as detectable expression in the lens, pineal gland, and brain. Within the interval between positions -205 and -185 is a region that contains two direct repeats of the hexamer, TGACCT. The proximal promoter binds three apparently retina-specific nuclear factors, Bp1, Bp2, and Bp3, through overlapping sequences centered between positions -25 and -15. Bp1 and Bp3 also recognize a closely related sequence found in the promoter regions of several other vertebrate photoreceptor-specific genes. Moreover, the consensus binding site for Bp1, designated PCE1, is identical to RCS1, an element known to play a critical role in eliciting photoreceptor-specific gene expression in *Drosophila melanogaster*. The results suggest that PCE1 and RCS1 are functionally, as well as structurally, similar and, that despite marked differences in the fly and vertebrate visual system, the transcriptional machinery involved in photoreceptor-specific gene expression has been strongly evolutionarily conserved.

Project Description

Objectives

The objective of this project is to understand the basic mechanism of phototransduction in the retina and to understand the structure, function, and evolution of the proteins present in photoreceptor rod cells and pinealocytes.

Methods

Conventional methods for analysis of proteins and nucleic acids are being used. These include protein purification, ribonucleic acid and deoxyribonucleic acid (DNA) isolation, characterization, and sequence determination. Various recombinant DNA techniques are also being used, including a Baculovirus expression vector system, synthesis of point mutation clones, characterization of promoters and transgenic animals. We have also synthesized and used purified oligopeptides and oligonucleotides.

Major Findings

(1) The gene sequences of S-antigen (S-Ag) from human and mouse were determined. It is 50 Kbp in length and has 15 introns and 16 exons. The smallest exon encodes for three amino acids.

(2) The intron-exon map sequence of the mouse S-Ag gene has been well conserved. Approximately 97 percent of the S-Ag gene is intron, and 3 percent is exon.

(3) The human and mouse S-Ag complementary DNAs (cDNA) have been subcloned into two expression vectors and have been expressed. The products of S-Ag cDNA were purified by column chromatography and were prepared for crystallization.

(4) The 5′-flanking sequence of the human and mouse S-Ag genes were determined and demonstrated promoter activity in the *in vivo* and *in vitro* transcriptional assays.

(5) Although the S-Ag promoter sequences are highly conserved between human and mouse, promoter activity was found at different locations of the 5′-flanking region in the human and mouse genes. This result suggests that the promoter activity is highly specific to tissues and species.

(6) The mouse S-Ag promoter, 1,300 bp in length, was fused with the chloramphenicol acetyl transferase (CAT) gene, and this gene was introduced into transgenic mice. The transgenic animals expressed CAT activity only in the retina and pineal gland. This result indicates that the promoters have a tissue-specific enhancer and promoter activity.

(7) The opsin promoter was fused with a diphtheria toxin gene. This fusion gene was introduced into transgenic mice, which subsequently lost only the photoreceptor rod cell layer.

(8) Several cDNAs of Shuzin, a retinal photoreceptor protein, were isolated from human and cow retinal cDNA libraries (lambda-gtll). The entire DNA sequences were determined. The deduced protein has sequence similarity with TFIID. Its gene was also isolated from a genomic library, and the DNA sequence was determined. It is comprised of two introns and three exons.

(9) Two genes of 33 kDa ROS-specific proteins have been isolated from the retinal libraries of human and mouse. The entire DNA sequence of these genes has been determined, and they have four exons and three introns.

(10) The proximal promoter sequence positions -38 to +304 are sufficient to direct low levels of retina-specific gene expression.

(11) The proximal promoter binds three retinal-specific nuclear factors, Bp1, Bp2, and Bp3, through overlapping sequences centered between positions -25 and -15.

(12) The distal promoter sequence positions -205 to -185 is a region that contains two direct repeats of the hexamer TGACCT.

(13) We found a consensus retinal photoreceptor-specific site (PCE1).

(14) The transcriptional machinery involved in photoreceptor-specific gene expression has been strongly and evolutionarily conserved.

Significance to Biomedical Research and the Program of the Institute

Eyes have remarkable properties in functioning efficiently over a wide range of illuminations. Rod cells having photosensitive rhodopsin are more sensitive to dim light and adapt in the dark to increase their sensitivity. However, rod cells cease their sensitive phototransduction in bright light. Cone cells in contrast do not operate in dim light but are operative in bright light. Rhodopsin, transducin, phosphodiesterase, rhodopsin kinase, and S-Ag have been known to be associated with the phototransduction cascade. Rhodopsin kinase and S-Ag are considered to be the important proteins for light-dependent modulation of phototransduction. To understand this light-dependent modulatory mechanism in rod outer segments, we have characterized S-Ag, Shuzin, and 33 K protein as well as their genes. Interestingly, other signal transduction systems have cascades similar to that of phototransduction (one of the best characterized receptor-mediated signal transduction processes). In the phototransduction cascade, the shutoff mechanism appears to be modulated by the phosphorylation and dephosphorylation of rhodopsin. Studying this modulation mechanism is important for understanding phototransduction as well as for understanding signal transduction in general. In addition, we think that the night blindness of vision may in part be associated with light adaptation.

Proposed Course

This project will be terminated.

NEI Research Program

Retinal Diseases—Photoreceptors and Pigment Epithelium

Publications

Abe T, Kikuchi T, Chang T, Shinohara T: The sequence of the mouse phosducin gene and its 5'-flanking region. *Gene* 133:179-186, 1993.

Abe T, Kikuchi T, Shinohara T: The sequence of the human phosducin gene and its 5' flanking region. *Genomic* 19:369-372, 1994.

Shinohara T, Kikuchi T, Tsuda M, Yamaki K: A family of retinal S-antigens (arrestins) and their genes: Comparative analyses of human, mouse, rat, bovine, and Drosophila. *Comp Biochem Physiol* 103:505-509, 1993.

Usukura J, Khoo W, Abe T, Shinohara T, Breitman M: Cone cells fail to develop normally in transgenic mice ablated of rod photoreceptor cells. *Tissue Cell* 275:79-90, 1994.

Laboratory of Sensorimotor Research

Report of the Chief
Laboratory of Sensorimotor Research

Robert H. Wurtz, Ph.D.

Research in the Laboratory of Sensorimotor Research concentrated on the control of movement by the visual system. These projects covered a range of topics extending from visual processing to the control of specific eye movements. My group has studied how visual motion filling the entire visual field (optic flow) might be organized to modify the movement of observers as they move through the visual world. Dr. Lance Optican looked at an earlier step in the visual pathway and investigated how visual information might be encoded in the patterning of neuronal impulses in visual cortex. Dr. Optican also developed a model of the control of rapid or saccadic eye movements based on recent work done in the laboratory on a brainstem structure, the superior colliculus (SC). Dr. David Lee Robinson investigated how these eye movements could be modified by changing activity in the colliculus during the generation of the eye movements. Dr. Michael E. Goldberg also studied saccadic eye movements but in relation to the question of how our spatial stability is maintained in spite of the eye movements that move our fixation from part of the field to another. Dr. Frederick A. Miles concentrated on a very different type of eye movement, the vergence eye movement, that allows us to fixate on objects at varying distances from us. A summary of each of the projects is presented below.

Section on Visual Motor Integration

While moving through the environment, the visual world streams past observers in a pattern depending on their movement. These optic-flow fields provide visual information that can guide self-motion, stabilize posture, and reveal the structure of the environment. My group has studied neurons in the dorsal region of the medial superior temporal area (MSTd) of monkey extrastriate cortex that previously have been shown to respond to the expanding radial motion that occurs with forward movement of an observer. Different directions of observer motion are accompanied by centers of motion located in different areas of the visual field, and, in our current experiments, we tested to see whether MSTd neurons responded best when centers of motion were located in different parts of the field. About 90 percent of the neurons studied responded differently to at least one stimulus with a shifted center of motion as compared with those positioned in the middle of the visual field. The preferred centers of motion were always limited to one area of the visual field for a given cell, and all parts of the visual field were represented. We found that many cells prefer a center of motion in the middle of the field, and those cells respond to motion over a more limited region of the field.

Using these experiments as a basis, we suggest that each of the MSTd neurons has a center of motion field with a gradient of preferred centers of motion and that there is an orderly arrangement of these neurons, with each region of the visual field being represented by a set of neurons. The responses of individual neurons would be graded according to proximity of the center of motion of a stimulus to the preferred center of motion for the neuron, just as other visual cortical neurons have gradients for the direction of planar motion or for the location of a spot of light. The role of MSTd neurons in interpreting the optic flow fields would not be one of qualitative feature matching but rather one of responding to visual motion according to the degree of match between the visual input and the preferred optic flow field of the neuron. These MSTd neurons could contribute to the determination of heading of observers moving through the environment. The organization of this area might also be relevant to the control of posture because the influence of full-field visual motion on posture is well recognized.

Section on Neural Modeling

In the past year, Dr. Optican's section has worked on two distinct problems. The first problem was how the brain represented visual information. The second problem was how the SC helped control saccadic eye movements.

Neuronal Encoding of Visual Parameters

Dr. Optican and his colleagues used a visual discrimination task to study the encoding of color and form information in cortical neurons. It has been proposed that color and form information is divided into separate channels (*e.g.*, cytochrome oxidase blob and interblob regions) in the cortex. A cluster method of information calculation was developed that made it possible to show that information about color and pattern rises over time in all neurons in cortical areas V1-V4. Such a result is not consistent with the idea that information about form and color is grouped into separate "channels" in cortex but rather suggests that all neurons participate in visual processing irrespective of the type of visual parameter involved.

Control of Two-Dimensional Saccadic Eye Movements

Recently, Drs. Douglas Munoz and Robert H. Wurtz identified three types of neurons (fixation, burst, and buildup) in the SC that were involved in controlling saccadic eye movements. During a saccade, activity seems to spread through the buildup neurons. Classic models, which control saccades in one dimension, have a resettable integrator within a local feedback loop. Dr. Optican has proposed a new, two-dimensional model of the saccadic system that places the SC inside the local feedback loop. His principal new hypothesis is that the spread of activity in the buildup neurons represents the resettable integrator of the local feedback loop.

One complication in this model is that the SC is not mapped in cartesian coordinates. Rather, there is a retinotopic map in polar coordinates that is warped logarithmically with eccentricity. Because polar coordinates do not follow the rules of vector addition, it is not simple to figure out how the activity in the buildup neurons should spread during the saccade to represent accurately the resettable integrator. Dr. Optican has shown that a vector field can provide the appropriate motor updates for the buildup neurons.

Section on Visual Behavior

Within the Section on Visual Behavior, Dr. David Lee Robinson and his colleagues have been studying the changes within the oculomotor pathways during the course of saccadic eye movements. Most models of the oculomotor system hypothesize that the signal for retinal error undergoes an integration before its effect on the oculomotor neurons. Our recent models have proposed that this integra-

tion actually takes place within the SC. By electrically stimulating the colliculus before and during eye movements, Dr. Robinson's group members have provided data that support this idea. They have demonstrated that stimulation at any time during periods of fixation leads to rather stereotyped eye movements; these have been termed fixed vector saccades because they are constant in terms of direction and amplitude.

In contrast, when the colliculus was stimulated during the course of a saccade, the directions of these evoked saccades were systematically shifted. Just as the eye began to move, the movement evoked by electrical stimulation was rotated in a direction opposite to the primary saccade by as much as 80 degrees. Excitation applied at progressively later times led to progressively smaller shifts. These data are of considerable importance in understanding the neural control of saccadic eye movements and specifying the collicular contributions to them.

Section on Neuro-Ophthalamologic Mechanisms

Dr. Goldberg and his collaborators have studied neurons in the lateral intraparietal that have visual responses. The visual responses are sometimes predictive: they occur before a saccade that will bring the spatial location of a visual target into the receptive field of the neuron. There are two possibilities to explain the phenomenon: In one, there is a general increase in retinal excitability, basically an expansion of the retinal receptive field; in the other, the receptive field moves on the retina, like a spotlight of excitability.

To distinguish between these two hypotheses, stimuli were flashed in the presaccadic receptive field or the postsaccadic receptive field at various times before and after the saccade. When a monkey is programming a saccade, neuronal excitability at the old receptive field decreases. Just before the saccade, many cells are unresponsive to visual stimuli that would be expected to drive them during normal fixation. At the same time, stimuli that would be expected to drive the neuron after the saccade drive the neuron before the saccade. This result suggests that the locus of excitability moves like a spotlight across the retina, so that the spatial location that will excite the cell after the movement excites it before the movement. The disappearance of excitability from the retinal location that will excite the neuron after the saccade prevents the cell from providing an inaccurate message after the saccade and enables spatially accurate processing across saccades.

Monkeys with deep cerebellar nuclear lesions have a battery of deficits in their oculomotor performance. The crude dynamics of their saccadic eye movements are normal; the main sequence of saccade peak velocity versus amplitude is normal, but the accuracy of visually guided saccades is diminished. Monkeys make larger saccades than normal, and this deficit is a function of initial orbital position. Normal monkeys can adjust the amplitude of saccades for target motion, but after deep cerebellar lesions monkeys do not compensate for target motion. They therefore relatively overshoot centripetally moving targets and relatively undershoot centrifugally moving targets. Normal monkeys can change the amplitude of their saccadic eye movements in response to intrasaccadic target steps; postlesion monkeys cannot. Finally, the variability of the amplitude of visually guided eye saccades is increased in the lesioned monkey. These data all suggest that the role of the cerebellum in the generation of saccadic eye movements is to fine-tune the transformation from visual stimulus to movement, a fine-tuning that requires an ongoing monitoring of the saccadic motor signal during the saccade.

Three patients with the unusual finding of torsional nystagmus during vertical pursuit were examined. Magnetic resonance imaging studies in these patients show small arteriovenous malformations in the region of the middle cerebellar peduncle. Eye movements recorded in one patient demonstrate torsional

nystagmus that scales linearly in velocity with increasing vertical pursuit velocity and is not present during saccades, horizontal pursuit, or horizontal vestibulo-ocular reflex cancellation. The torsional velocity is decreased when the patient pursues an afterimage target, a case where there is no retinal slip signal present. These findings suggest that the problem in these patients is in the visual-to-motor-signal coordinate transformation. The linear relationship between torsional and vertical pursuit velocity suggests that these findings arise from an inappropriate cross-coupling between vertical visual (or motor) signals, which are used in vertical pursuit, and the torsional eye movement system.

Section on Oculomotor Control

Using monkeys, Dr. Miles and his collaborators have demonstrated that the vergence eye movements resulting from sudden changes in the binocular disparity of large textured scenes have almost machine-like consistency and a latency of 52-53 msec, which is less than one-third of the value commonly cited in the literature (which was obtained with small targets). They suggest that these disparity vergence responses are mediated by neurons that have binocular receptive fields that are spatially selective (in terms of size, shape, and orientation) and offer new insights into the properties of such (cortical) neurons. These short-latency-vergence eye movements were strongly affected by an antecedent saccadic eye movement, whereby disparity steps applied in the immediate wake of a saccade were much more effective than identical steps delivered only 200 milliseconds later: transient postsaccadic enhancement. This postsaccadic enhancement should normally help to speed binocular realignment when gaze is redirected to (large?) objects in different depth planes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00045-16 LSR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Visuomotor Properties of Neurons in the Thalamus

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	David Lee Robinson	Ph.D.	Section Chief	LSR, NEI
Others:	Alexander A. Kustov	Ph.D.	Visiting Fellow	LSR, NEI

COOPERATING UNITS (*if any*)

Department of Anatomy, Howard University (Robert J. Cowie, Ph.D.)

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Visual Behavior Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.3	2.0	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

With each movement of the eyes, there are changes in the state of the neural processes that generate eye movements and changes in the perceptual frame of reference. We have electrically stimulated the superior colliculus at various times around visually guided eye movements to understand the collicular contribution to these changes. When the colliculus is stimulated during periods of fixation, the amplitude and direction of the saccadic eye movement is rather constant. When the same stimulation is applied while an eye movement is in progress, there is a systematic shift in the direction of the evoked eye movement. Just as the eye begins to move toward a visual target, the eye movement evoked by stimulation of the colliculus is rotated in a direction opposite to the primary visual saccade by as much as 80 degrees. Electrical stimulation that is applied at progressively later times during the visually guided eye movement will evoke eye movements with progressively less shift. These data suggest that the colliculus participates in the changes in spatial reference during eye movements or that there is a gradual decay in the integration process that the colliculus provides to the oculomotor system.

Project Description

Objectives

Within the oculomotor pathways, there are changes before, during, and after each movement of the eyes. These are related to the underlying neural processes that generate the movement, and they are related to the planning that must take place before the next eye movement. It has been known for a number of years that the superior colliculus (SC) plays an important role in the generation of saccadic eye movements. The present studies were conducted to understand how the colliculus relates to the preparation for saccadic eye movements and the compensation for neural computations after their initiation.

Methods

To localize specific regions of the brain and reliably test these sites, adult monkeys were trained to enter a primate chair, sit quietly, and fixate on a spot of light. They also learned to make rapid, saccadic eye movements when the fixation spot was turned off and another light appeared. The timing between these two events was varied to change the temporal properties of the eye movements and also modify the direction of the animal's attention. After the animals were adapted to these situations, they were implanted during sterile surgery with several devices for recording eye movements, electrical activity within the brain, and instruments for head restraint. Once the monkeys had recovered from the surgery, we positioned fine wire electrodes into specific brain sites, recorded the discharge patterns of individual cells, and electrically excited these loci. The timing of the electrical stimulation was varied in relation to the onset of target lights as well as the time course of the evoked saccadic eye movements. The evoked eye movements and other behavioral responses were recorded and quantified.

Major Findings

As the oculomotor system plans and generates each eye movement, there are many changes within its pathways and those systems that begin to calculate the next saccade. We wanted to learn how the SC interacted with these changing requirements for saccade programming.

When an electrode enters the superficial layers of the SC, the first neurons encountered are very visually responsive. These neurons have relatively small visual receptive fields that have a fixed position relative to the fovea. The location of the receptive field moves with the direction of the eye. At these dorsal sites within the colliculus, the threshold for evoking saccadic eye movements by electrical stimulation is at its highest (100 μA). If stimulation of these cells occurs while the monkey fixates a central target, then the evoked eye movement has a fixed vector; the eye moves in a set direction with a set amplitude. If the stimulation is applied while the monkey fixates targets in different locations, then there is a slight effect of the initial eye position on the evoked eye movement. Eye movements that start on the side of the stimulating electrode are slightly larger than those that start opposite the stimulation site.

Next, we wanted to learn if the planning or execution of an eye movement would alter the movement evoked by electrical stimulation. If the same site were stimulated during fixation, after the disappearance of the fixation point, or shortly after the appearance of the eye movement target, then the same, fixed vector saccade was also elicited. Eye movements that were evoked by stimulation just before the visually guided saccade had a slight shift in their evoked direction. If the electrical stimulation was applied during the course of a visually guided eye movement, then the threshold (120 μA) and latency were increased. The characteristics of these evoked saccades were also modified but will be considered in a later section.

As we advanced the microelectrode deeper within the SC, we reached a transition point where the neurons began to respond with a brisk burst of activity that was before and time-locked with the saccadic eye movement. The amount of visual excitability of these neurons was less than for the cells above them. When we electrically stimulated at the sites of these cells, the threshold was much less (20 μA). In addition, the latency of the evoked eye movements was less; they began at least 15 milliseconds (msec) sooner. Just as for the more dorsal sites, the eye movements evoked during periods of fixation had fixed vectors, and there was the same subtle influence of initial eye position. Stimulation applied at points long before the visually guided saccade evoked the same reproducible eye movement. Eye movements that were evoked by stimulation just before the visually guided saccade to a contralateral target had a slight shift in direction.

When we stimulated the colliculus at the sites of eye movement cells during the course of an eye movement, the threshold did not increase as it had at the more dorsal, visual sites. Also, the latencies of the movements were shorter. The directions of these eye movements shifted in a systematic fashion. At the instant of the start of the eye movement to a visual target, the evoked eye movements had a direction that was shifted from the fixed vector by as much as 90 degrees; the shifted direction was opposite to that of the primary visual saccade. As the time of stimulation occurred later and later during the visually guided saccade, the amount of rotation from the fixed vector systematically diminished. The final position of these electrically evoked saccades was on a line parallel to the plane of the visually guided eye movements. The time period during which the evoked saccades were shifted lasted about 80 msec longer than the duration of the executed visually guided saccade. Thus, the absolute duration of the whole process could last as long as 120 msec.

As we advanced the microelectrode still deeper, we encountered neurons that had more prolonged activity between the appearance of the saccade target and the saccade to acquire it. Electrical stimulation here evoked fixed vector saccades during periods of fixation, slight influences due to initial position, latencies and thresholds similar to the sites just above, and systematic shifts in the direction of movements when they were evoked during the course of the eye movement.

These data show that the eye movements elicited by electrical stimulation are systematically influenced by the preparation and occurrence of eye movements. There are two possible mechanisms for these effects. The direction of gaze changes with each eye movement, and the visual scene is also modified; thus, there is the need to establish a new frame of reference. The systematic shift in evoked eye movements may reflect this slower change in the oculomotor frame of reference. Certain studies of spatial localization during the course of saccadic eye movement provide perceptual data to support this interpretation. Alternatively, the systematic shift may reflect the resetting of the neural integrator. In most models of the oculomotor system, it has been hypothesized that the signal for the retinal error is integrated before its effect on the motoneurons of the eye muscles. After this integration has taken place, the neural elements responsible will take some time to return to their state of rest. Recent data suggest that some neurons within the SC participate in the integration; the systematic shift in electrically evoked saccades may reflect the resetting of the integrative functions of these neurons.

Significance to Biomedical Research and the Program of the Institute

One of the major goals of system neuroscience is to understand how the brain controls movement. A thorough understanding of this function of the brain is necessary for any attempts to rehabilitate patients with central nervous system lesions. Considerable progress has been made in this area from studies of the control of the oculomotor system. The SC is known to be a major participant in the transformation of visual signals to the oculo-

motor system. The ideas presented here help to clarify the types of signals that the colliculus sends to the oculomotor system and the impact of these signals on the control of saccadic eye movements.

Proposed Course

The most studied aspect of the SC is the presaccadic discharge of neurons within its intermediate layers. Most modeling of this activity describes descending SC projections to preoculomotor centers. However, previous anatomical data have shown that this region also projects to the thalamus. Preliminary data obtained while extending our study of collicular control of eye movement have demonstrated that the region of the thalamus that receives a projection from the collicular burst neurons contains cells that discharge before eye movements. Our future studies will involve extensive recording from these neurons, characterizing their discharge patterns, testing the effects of microstimulation of these cells, and evaluating the effects of localized inactivations of this region.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Visual Processing and Functional Organization, Structure and Function of Central Visual Pathways

Publications

Cowie RJ, Robinson DL: Head and eye movements elicited from the superior colliculus of the macaque. *Soc Neurosci Abstr* 18:788, 1993.

Cowie RJ, Robinson DL: Subcortical contributions to head movements in macaques. I. Contrasting effects of electrical stimulation of a medial pontomedullary region and the superior colliculus. *J Neurophysiol*, in press.

Cowie RJ, Smith MK, Robinson DL: Subcortical contributions to head movements in macaques. II. Connections of a medial pontomedullary head-movement region. *J Neurophysiol*, in press.

Kustov AA, Robinson DL: Changes in saccade trajectory from stimulation of the colliculus of the macaque. *Soc Neurosci Abstr* 20(1):141.

Robinson DL, Bowman EM, Kertzman C: Covert orienting of attention in macaques. II. Contributions of parietal cortex. *J Neurophysiol*, in press.

Robinson DL, Cowie RJ: Attentional engagement and the pulvinar. *Behav Brain Sci* 16:586-587, 1993.

Robinson DL, Cowie RJ: Influences of subcortical centers on head movements, in Buttner U, Brandt T, Fuchs A, Zee D (eds): *Contemporary Ocular Motor and Vestibular Research: A Tribute to David A. Robinson.* International Meeting Eibsee, 1993. Stuttgart, New York, Georg Thieme Verlag and New York, Thieme Medical Publishers.

Robinson DL, Cowie, RJ: Visual contributions of the pulvinar, in McCormick DA, Jones EG, Steriade M (eds): *Thalamus.* Elsevier Science Ltd., in press.

Robinson DL, Kertzman C: Covert orienting of attention in macaques. III. Contributions of the SC. *J Neurophysiol*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00049-16 LSR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Cerebral Cortical Mechanisms for Eye Movements and Visual Attention

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others:	Edmond J. FitzGibbon	M.D.	Medical Officer	LSR, NEI
	Carol L. Colby	Ph.D.	Senior Staff Fellow	LSR, NEI
	Makoto Kusunoki	Ph.D.	Visiting Fellow	LSR, NEI
	Marc Umeno	M.A.	pre IRTA	LSR, NEI

COOPERATING UNITS (*if any*)

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Neuro-Ophthalmologic Mechanisms Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.2	4.0	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Three lines of inquiry were followed to determine how the cerebral cortex and its efferent regions control eye movements and visuospatial attention.

In the first single, a neuron recording was used to probe the mechanisms whereby the parietal cortex of the monkey achieves spatial accuracy. It is well known that parietal neurons respond to stimuli in a certain location on the retina, the receptive field. The receptive fields of parietal neurons transiently change before a saccadic eye movement, so that they respond, before the eye movement, to stimuli that will be in their receptive fields after the movement. At the same time, the current retinal location becomes unresponsive. These data establish that there is a specific shift of retinal receptive field, rather than a general change of excitability.

In the second, the oculomotor performance of rhesus monkeys was assessed while the monkeys performed an oculomotor task before and after electrolytic lesions of the deep cerebellar nuclei. A number of deficits were found that were consistent with the cerebellum playing a role in adjusting the amplitude of saccadic eye movements but not in initiating them: monkeys had increased variability in the amplitude of their saccadic eye movements, they had tended to overshoot targets and this inaccuracy was dependent upon the initial position of the eye in the orbit; they were unable to adjust the amplitude of their saccadic eye movements for target motion; and they could not adjust the amplitude of their saccadic eye movements in a model of muscle weakness.

In the third, the performance of humans with superior cerebellar peduncle lesions was studied in a smooth pursuit task. Cross-coupling between the torsional and vertical pursuit systems was demonstrated.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

This section has concentrated on three aspects of the physiology and phenomenology of higher visual and oculomotor processing in monkey and human, especially as these functions relate to the parietal and frontal regions of the cerebral cortex, their afferent regions, and their efferent targets. Previous work in this section has shown that neurons in the parietal cortex discharge in response to visual stimuli and before saccadic eye movements. The neurons contain a predictive aspect to their visual responses: neurons respond to stimuli whose spatial location will be brought into their visual receptive fields by impending saccadic eye movements. Work in the laboratory for this period has concentrated on understanding the mechanism of this predictive response by studying the time course of changes in retinal excitability before and after saccadic eye movements.

Although the cerebellum has been known to participate in the guidance of saccade eye movements, its exact contribution has not been clear. To establish the role of the cerebellum in the guidance of saccadic eye movements, and to chart a path for future research, comprehensive lesions were made in the deep cerebellar nuclei of rhesus monkeys; the performance of these monkeys was studied in a number of tasks before and after the lesions.

Human disease can often provide insights into normal processes. In the course of evaluating oculomotor performance in patients with neurological disease, three patients were discovered with torsional nystagmus on upward smooth pursuit. All three had lesions of the superior cerebellar peduncle. The magnetic search coil technique was used to study their eye movements quantitatively.

Methods

Monkeys were implanted with magnetic search coils for the measurement of eye position, along with devices for temporary restraint and electrophysiological recording and stimulation. They were trained to perform a number of visuomotor tasks, including fixation, saccades, and smooth pursuit. Microelectrodes were placed in the lateral intraparietal area, and single neurons were studied while the monkey performed various visuomotor tasks. In a second set of experiments, microelectrodes were placed in the deep cerebellar nuclei of the monkeys, neurons recorded, and saccades evoked by electrical stimulation. Electrolytic lesions were made by passing current through the recording microelectrodes until saccades could no longer be evoked from the area. Oculomotor performance was evaluated after the lesion, using the same battery of tasks for which normal data had been established before the lesion.

Magnetic search coil limbic rings were placed on one patient with torsional nystagmus during vertical smooth pursuit, and his eye movements were measured while he performed pursuit eye movements at several velocities.

Major Findings

Neurons in the lateral intraparietal have visual responses. The visual responses are sometimes predictive: they occur before a saccade that will bring the spatial location of a visual target into the receptive field of the neuron. There are two possibilities to explain the phenomenon: in one, there is a general increase in retinal excitability, basically an expansion of the retinal receptive field; in the other, the receptive field moves on the retina, like a spotlight of excitability. To distinguish between these two hypotheses, stimuli were flashed in the presaccadic receptive field or the postsaccadic receptive field at various times before and after the saccade.

When a monkey is programming a saccade, neuronal excitability at the old receptive field decreases. Just before the saccade, many cells are unresponsive to visual stimuli that would be expected to drive them during normal fixation. At the same time, stimuli

that were expected to drive the neuron after the saccade drive the neuron before the saccade. This result suggests that the locus of excitability moves like a spotlight across the retina, so that the spatial location that will excite the cell after the movement excites it before the movement. The disappearance of excitability from the retinal location that will excite the neuron after the saccade prevents the cell from providing an inaccurate message after the saccade and enables spatially accurate processing across saccades.

Monkeys with deep cerebellar nuclear lesions have a battery of deficits in their oculomotor performance. The crude dynamics of their saccadic eye movements are normal; the main sequence of saccade peak velocity versus amplitude is normal, but the accuracy of visually guided saccades is diminished: monkeys make larger saccades than normal, and this deficit is a function of initial orbital position. Normal monkeys can adjust the amplitude of saccades for target motion, but, after deep cerebellar lesions, monkeys do not compensate for target motion, and therefore relatively overshoot centripetally moving targets and relatively undershoot centrifugally moving targets. Normal monkeys can change the amplitude of their saccadic eye movements in response to intrasaccadic target steps; postlesion monkeys cannot. Finally, the variability of the amplitude of visually guided eye saccades is increased in the lesioned monkey. These data all suggest that the role of the cerebellum in the generation of saccadic eye movements is to fine-tune the transformation from visual stimulus to movement, a fine-tuning that requires an ongoing monitoring of the saccadic motor signal during the saccade.

Three patients with the unusual finding of torsional nystagmus during vertical pursuit were examined. Magnetic resonance imaging studies in these patients show small arteriovenous malformations in the region of the middle cerebellar peduncle. Eye movements recorded in one patient demonstrate torsional nystagmus that scales linearly in velocity with increasing vertical pursuit velocity and is not present during saccades, horizontal pursuit, or horizontal vestibulo-ocular reflex cancellation. The torsional velocity is decreased when the patient pursues an afterimage target, a case where there is no retinal slip signal present. These findings suggest that the problem in these patients is in the visual-to-motor-signal-coordinate transformation. The linear relationship between torsional and vertical pursuit velocity suggests that these findings arise from an inappropriate cross-coupling between vertical visual (or motor) signals that are used in vertical pursuit and the torsional eye movement system.

Significance to Biomedical Research and the Program of the Institute

Understanding how the cerebral cortex and its afferent regions guide eye movements and modulate visual attention and learning is useful both as a model for the neural control of other, more complicated behaviors and as a key to understanding and developing treatments for disorders of the neural control of vision, eye movements, and attention.

Proposed Course

Lesions in the superior colliculus will be made in monkeys with deep cerebellar lesions, to see if the deficit previously seen in monkeys with combined frontal and collicular lesions can be duplicated in monkeys with combined cerebellar and collicular lesions. Frontal eye field and parietal neurons will be studied in a paradigm that more closely resembles a normal visual environment, to see if the activity demonstrated in a sparse visual environment exists under more normal circumstances.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Visual Processing and Functional Organization, Structure and Function of Central Visual Pathways

Publications

Colby CL, Duhamel JR, Goldberg ME: Oculocentric spatial representation in parietal cortex. *Cerebral Cortex*, in press.

Goldberg ME, Musil SY, Colby CL, Duhamel JR, Olson, CR: Cortical mechanisms for voluntary and involuntary attention: Posterior cingulate and lateral intraparietal areas in the monkey, in Albowitz B, Albus K, Kuhnt U, Nothdurft HC, Wahle P (eds): *Structural and Functional Organization of the Neocortex.* New York, Springer-Verlag, 1994, pp 267-278.

Olson CR, Musil SY, Goldberg ME: Posterior cingulate cortex and visuospatial cognition: properties of single neurons in the behaving monkey, in Vogt BA, Gabriel M (eds): *Neurobiology of Cingulate Cortex and Limbic Thalamus: A Comprehensive Handbook.* Boston, Birkhauser, 1993, pp 366-380.

Segraves MA, Goldberg ME: Effect of stimulus position and velocity upon the maintenance of smooth pursuit eye velocity. *Vision Res*, in press.

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PROJECT NUMBER

Z01 EY 00153-12 LSR

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visual Motion and the Stabilization of Gaze

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Frederick A. Miles	D.Phil.	Senior Research Physiologist	LSR, NEI
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	Geoffrey A. Bush	Ph.D.	IRTA Fellow	LSR, NEI
	Claudio Busettini	Ph.D.	Visiting Scientist	LSR, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Oculomotor Control Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.8	2.6	1.2

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using monkeys, we have demonstrated that the vergence eye movements resulting from sudden changes in the binocular disparity of large textured scenes have almost machine-like consistency and a latency of 52-53 msec, which is less than one-third of the value commonly cited in the literature (which was obtained with small targets). For small steps (less than about 2 degrees) initial vergence accelerations were generally in the correct direction—convergent with crossed disparities and divergent with uncrossed—and the initial vergence acceleration increased with increases in disparity. However, as disparities exceeded about 2 degrees, initial vergence accelerations gradually regressed to a low, albeit nonzero (convergent) level. In fact, disparities in excess of 4 to 5 degrees—regardless of whether crossed, uncrossed, or vertical—resulted in the same initial (weakly convergent) responses. This was not due to an esophoria or to accommodative convergence because there were no early vergence responses when the image seen by the left eye was blanked rather than simply shifted. Further, these default convergence responses were not simply due to the disruption of retinal image stability consequent to shifting the retinal images because they were not seen with conjugate steps: they must have resulted specifically from the change in binocular disparity. That responses peaked with disparities of about 2 degrees (or less) suggests that the neurons providing the primary drive for these early vergence responses only decode small disparities. We suggest that these disparity vergence responses are mediated by neurons that have binocular receptive fields that are spatially selective (in terms of size, shape, and orientation) and offer new insights into the properties of such (cortical) neurons. The default responses might represent the net output of such neurons to uncorrelated patterns, a phenomenon recently observed in monkey visual cortex.

These short-latency-vergence eye movements were strongly affected by an antecedent saccadic eye movement, whereby disparity steps applied in the immediate wake of a saccade were much more effective than identical steps delivered only 200 ms later: transient postsaccadic enhancement. Further, this enhancement was due in part to the visual stimulation elicited by the saccadic eye movement and could be simulated by shifting the visual scene in a saccade-like way. We suggest that this postsaccadic enhancement will normally help to speed binocular realignment when gaze is redirected to (large?) objects in different depth planes.

Project Description

Objectives

Good binocular vision requires that the two eyes be aligned on the same object, and this necessitates disconjugate (vergence) eye movements. Neurons that sense the slight differences in the images seen by the two eyes—resulting from the slight difference in their viewpoints ("binocular disparity")—are thought to have an important role in this process. The advent of new techniques for recording eye position with high precision in both monkeys and humans has for the first time permitted detailed studies of the relative alignment of the two eyes on objects at varying distances in a three-dimensional world. We have used these high-resolution techniques (the electromagnetic search coil) to examine the vergence eye movements that result from sudden changes in the binocular disparity of a large, textured scene (more than 40 degrees x 40 degrees).

Methods

Subjects (three rhesus monkeys) faced a tangent screen onto which were projected two superimposed images, each of which was visible only to one of the two eyes. This was achieved using a special screen and polarizing filters so that one of the images was polarized in a plane orthogonal to the other, and, when viewed through goggles with cross-polarizing filters, each eye saw only one of the images. The images were irregular geometrical forms or computer-generated random dot patterns. The positions of both eyes were recorded using the electromagnetic search coil method, and disparity steps were applied to the patterned background after subjects made saccadic eye movements from a target located 10 degrees right of center to one straight ahead. The targets were extinguished during the centering saccade so that only the background patterns were visible when the eyes arrived at the center of the screen.

Major Findings

The vergence eye movements resulting from sudden changes in the binocular disparity of a large textured scene had almost machine-like consistency and ultrashort latency: 52-53 millisecond (msec). This is less than one-third of the latency commonly cited in the literature (about 160 msec). Most previous studies have used small targets viewed against a dark or featureless background, necessitating that animals be trained to perform appropriately. This is not necessary with large textured scenes: Responses were obligate, and monkeys were neither trained to track the steps nor reinforced for doing so. For small steps (less than about two degrees), initial vergence accelerations were generally in the correct direction—convergent with crossed disparities and divergent with uncrossed—and the initial vergence acceleration increased with increases in disparity. However, as disparities exceeded about two degrees, initial vergence accelerations gradually regressed to a low, albeit nonzero (convergent) level. In fact, disparities in excess of four to five degrees, regardless of whether crossed, uncrossed, or vertical, resulted in the same initial (weakly convergent) responses. This was not due to an esophoria or to accommodative convergence because there were no early vergence responses when the image seen by the left eye was blanked rather than simply shifted. Further, these default convergence responses were not simply due to the disruption of retinal image stability consequent to shifting the retinal images because they were not seen with conjugate steps: They must have resulted specifically from the change in binocular disparity. We suggest that the default responses represent the net output of spatially tuned disparity detectors to uncorrelated patterns, a phenomenon recently observed in monkey visual cortex.

These short-latency-vergence eye movements were strongly affected by an antecedent saccadic eye movement, whereby disparity steps applied in the immediate wake of a saccade were much more effective than identical steps delivered only 200 msec later: tran-

sient postsaccadic enhancement. Further, this enhancement was due in part to the visual stimulation elicited by the saccadic eye movement and could be simulated by shifting the visual scene in a saccade-like way. The postsaccadic enhancement of disparity vergence resembles the postsaccadic enhancement of early ocular following that we described some years ago. We suggest that this enhancement will normally help to speed binocular realignment when gaze is redirected to large objects in different depth planes.

We have recently obtained preliminary data that indicate that humans also show short-latency disparity vergence responses with large textured patterns: The mean latency (±SD) for five subjects was 79 (±5) msec, which is almost exactly one-half the value usually cited in the literature. Human disparity vergence also showed a limited range of sensitivity to disparity, with default (convergence) responses for disparities exceeding a few degrees, regardless whether crossed, uncrossed, or vertical, as well as postsaccadic enhancement.

Significance to Biomedical Research and the Program of the Institute

Despite their ultrashort latency, it is highly likely that these vergence eye movements are mediated by cortical pathways because binocular disparity is thought to originate from cortical processing. (It has been reported that a human subject with a sectioned corpus callosum could not initiate vergence eye movements to images presented to opposite hemispheres, that is, to objects bounded by the two lines of sight. This points to the cortical mediation of vergence eye movements in general, not merely disparity-driven vergence.) That responses peaked with disparities of approximately two degrees (or less) suggests that the neurons providing the primary drive for these early vergence responses only decode small disparities. We suggest that the disparity of extensive patterns such as those that we have used can only be sensed by neurons that have binocular receptive fields that are spatially selective (in size, shape, and orientation). These short-latency vergence eye movements elicited by large textured patterns provide a new tool for examining the temporospatial properties of spatially selective binocular cortical neurons. That the responses were enhanced by an antecedent saccade or saccade-like shift of the field points to a visually mediated boost of vergence at the very time that binocular alignment is commonly most challenged—in the wake of saccades to a new target in a new plane.

Proposed Course

Future studies will examine the temporospatial properties of these short-latency vergence eye movements in monkeys and humans.

NEI Research

Strabismus, Amblyopia, and Visual Processing—Image Formation and Stabilization, Ocular Motility

Publications

Busettini C, Krauzlis RJ, Miles FA: Short-latency vergence responses, in Buttner U, Brandt T, Fuchs AF, Zee DS (eds): *Contemporary Ocular Motor and Vestibular Research: A Tribute to David A. Robinson.* International Meeting Eibsee, 1993. Stuttgart, New York, Georg Thieme Verlag and New York, Thieme Medical Publishers.

Busettini C, Miles FA, Schwarz U, Carl JR: Human ocular responses to translation of the observer and of the scene: dependence on viewing distance. *Exp Brain Res,* in press.

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Kawano K, Inoue Y, Takemura A, Miles FA: Effect of disparity in the peripheral field on

short-latency ocular following responses. *Visual Neurosci* 11:833-837, 1994.

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Miles FA: The sensing of optic flow by the primate optokinetic system, in Findlay JM, Kentridge R (eds): *Eye Movement Research Mechanisms, Processes and Applications.* New York, Elsevier, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00109-14 LSR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visuomotor Processing in the Primate Brain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others:	Hiroshi Aizawa	Ph.D.	Visiting Fellow	LSR, NEI
	Gregg H. Recanzone	Ph.D.	Guest Researcher	LSR, NEI
	Charles J. Duffy	M.D., Ph.D.	Senior Staff Fellow	LSR, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Visuomotor Integration Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.6	3.2	1.4

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither
[ ] (a1) Minors
[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Neurons in the dorsal region of the medial superior temporal area (MSTd) of monkey extrastriate cortex have previously been shown to respond to the expanding radial motion that occurs as an observer moves through the environment. In previous experiments, the MSTd neurons have been shown to respond to radial and circular motion when the center of that motion was in the middle of the visual field. Different directions of observer motion are accompanied by centers of motion located in different areas of the visual field, and in our current experiments we tested to see whether MSTd neurons responded best when centers of motion were located in different parts of the field. About 90 percent of the 245 neurons studied responded differently to at least one stimulus with a shifted center of motion as compared with those positioned in the middle of the visual field. The preferred centers of motion were always limited to one area of the visual field for a given cell, and all parts of the visual field were represented. There was a preference among the sample of neurons for centers of motion located closer to the middle of the visual field, and neurons preferring this part of the field also responded to centers of motion over a more limited region of the field. Based on these observations, we suggest that each of the MSTd neurons has a center of motion field with a gradient of preferred centers of motion and that there is an orderly arrangement of these neurons with each region of the visual field being represented by a set of neurons. This neuronal organization creates the potential for graded responses from individual neurons for different directions of heading as an observer moves through the environment.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

We have continued our studies of the visual control of movement through the investigation of several questions about the organization of the cerebral cortex and brainstem of the rhesus monkey. This year, we have studied the control of saccadic eye movements by the brainstem (the superior colliculus), the modulation of visual processing by attention in extrastriate areas of cortex, and the role of activity in extrastriate areas in providing visual information concerning the heading of an observer moving through the environment. This report summarizes work on this latter topic.

While moving through the environment, the visual world streams around observers in a pattern that reflects their motion. These optic-flow fields combine the effects of all observer movements in three-dimensional space to provide visual information that can guide self-motion, stabilize posture, and reveal the structure of the environment. Previous efforts to investigate the neuronal processing related to this optic flow has concentrated on the medial superior temporal area (MST) of the monkey cerebral cortex. Neurons in this region respond to visual motion, and neurons in the dorsal part of this area (MSTd) have large receptive fields that are most responsive to the movement of correspondingly large patterns of motion. Previous work in our laboratory has demonstrated that many of these neurons respond selectively to components of optic flow stimuli, including radially expanding stimuli. In addition, in our last annual report, we provided evidence that monkeys use the optic flow stimuli in the stabilization of their posture.

In our previous experiments, and in those of others, we have generally tested the neurons to radial motion when the center of motion was in the center of the monkey's visual field. One possibility is that these neurons might respond better if that center of motion were shifted to different regions of the visual field, as would be the case if the observer were moving in different directions in the visual field under certain conditions. To test this possibility, we studied the MSTd neurons with large-field optic flow stimuli with centers of motion shifted to different regions of the visual field.

Methods

Our experiments use the rhesus monkey, *Macaca mulatta*, for three reasons. First, our experiments frequently require the monkey to perform specific behaviors in the course of the experiments, and the monkey is easily trained and becomes a cooperative subject in experiments requiring high levels of visual performance. Second, to study the cells related to such a complex function as optic flow, we need to know where the characteristics appropriate for analysis of this type of visual motion are in the brain cells. The extensive study of the cerebral cortex in the rhesus monkey over the past two decades and the identification of specific areas that contain the appropriate neurons, make it possible to study the phenomenon of optic flow. Third, the behavior in response to optic flow in the monkey is similar to that studied extensively in humans, and what is learned in the monkey is directly relevant to problems in humans.

An online computer controls the experiment; it rewards the monkey, collects the neuronal and behavioral data, and stores these data for later analyses. A second computer, under control of the first, generates the optic flow stimuli that are projected on a screen in front of the monkey by a television projector. During the experiment, the monkey faces the screen and fixates on a small spot of light in different areas of the screen for a period of several seconds to obtain a reward.

Major Findings

We studied the responses of all neurons we encountered in the MSTd area and concentrated on 245 cells that responded to radial and circular motion, with the center of this motion

in the middle of the visual screen. We next tested each of these cells with the stimulus to which it responded best, radial motion or circular motion, with the center of motion moved away from the middle of the screen. We found that about 90 percent of the neurons had significantly different responses to one or more of these shifted centers of motion.

Thus, a large fraction of the neurons gave a different response to the stimulus with a shifted center of motion than they did to the centered radial or circular stimuli, although the magnitude of the response changes varied widely. We found a distribution of neuron types. Some showed the best response to the centered motion in the middle of the visual field with all other responses being significantly smaller; some showed the best responses to a center of motion that was eccentric from the center; others responded best to a planar stimulus, one that we regarded as having a peripheral center of motion off the screen. All the areas of the visual field that we tested (17 sites) had at least one neuron that responded to a center of motion in that area, so that we conclude that the population of neurons in MSTd are likely to be capable of responding to a wide variety of different centers of motion.

The most frequently preferred individual stimulus was one centered in the middle of the visual field. Furthermore, when we examined over what region of the field a given cell would respond to centers of motion, we found that neurons preferring a center of motion in the middle of the field responded to centers of motion over the smallest regions of the field. Thus, many cells prefer a center of motion in the middle of the field, and those cells respond to motion over a more limited region of the field. In contrast, fewer cells prefer centers of motion that are more eccentric from the center; when they prefer such centers, they prefer the centers over a much larger region of the field, frequently a quadrant of the visual field.

In our complete survey of neurons, we used 17 stimuli with different centers of motion randomly presented. On a smaller number of neurons we used more stimuli (25 or more), and with this finer grain analysis for the centers of motion, we found neurons that selectively preferred the location of the added centers of motion that we presented. These studies with more refined stimuli suggest that the preferred center of motion, rather than being a simple segment of the visual field, has a more complex structure that our relatively limited stimuli are only beginning to tap.

Thus, we found that MST neurons are sensitive to shifts in the centers of motion: at least one of the shifted center of motion stimuli produced a response significantly different from the centered stimulus in more than 90 percent of the neurons. More than one-half of our neurons gave significant responses to three or fewer stimuli, indicating that the preferred centers of motion were clustered in one part of the visual field and not scattered about. The neurons studied preferred centers of motion throughout all parts of the visual field, with the largest number preferring centers of motion in the middle of the field. These neurons responded to centers of motion over a more limited area of the field. These observations suggest that a center of motion and the accompanying shift in the pattern of that motion throughout the visual field are stimuli characteristics to which the MST neurons respond.

We suggest that each of the MSTd neurons can be regarded as having a preferred center of motion. The responses of individual neurons would be graded according to proximity of the center of motion of a stimulus to the preferred center of motion for the neuron, just as other visual cortical neurons have gradients for the direction of planar motion or for the location of a spot of light. The role of MSTd neurons in interpreting the optic flow fields would not be one of qualitative feature matching but rather one of responding to visual motion according to the degree of match between the visual input and the preferred optic flow field of the neuron. The population of neurons would have a gradient in the density of the centers of motion with

those near the middle of the field more highly represented and responding to centers of motion over a more limited area of the field.

Significance to Biomedical Research and the Program of the Institute

We think that the MSTd neurons that respond to radial motion with centers of motion arrayed across the visual field could contribute to the determination of heading of observers moving through the environment. Although heading may be determined by a combination of visual cues, our findings support the conclusions of a number of psychophysical experiments that suggest the existence of brain mechanisms that might determine heading from optic flow. The simplification in our visual stimuli limit the conditions under which we can apply our observation to heading judgments. But within those limits, the activity of the cells we observed could be used to determine heading. Their activity would be relevant, for example, in determining heading with a fixation point at a distance, as in a visual landing of an airplane where the optic flow conditions closely approximate the conditions in our experiment.

Our experiments are also relevant to a growing number of neural models that attempt to show how directionally sensitive elements in the brain might produce heading judgments.

Although we have concentrated in our discussion on the relevance of these neurons for influencing the heading of an observer moving through the environment, the organization of this area is also relevant to the control of posture. Such full-field stimulus control of posture is well recognized, and the visual control of posture is an area only recently receiving more intense investigation. An understanding of the neuronal mechanisms underlying the visual processing is essential for full understanding of how vision modulates posture.

Proposed Course

In the experiments described, the optic flow stimuli were deliberately made as simple as possible to allow study of as many neurons as possible and to have as simple an interpretation as possible. Future experiments are needed to expand the nature of these stimuli to more closely approximate those encountered in the environment, particularly by including depth in the stimulus instead of the single-plane stimuli used in the present experiments. In addition, experiments in this laboratory have shown that many of the cells studied in the present experiments are also sensitive to binocular disparity, that is, they give information by binocular mechanisms about depth just as optic flow gives information about movement. Combining experiments using both binocular depth information and optic flow information might reveal a sensitivity of these neurons beyond what these initial experiments have revealed.

NEI Research Program

Strabismus, Ambylopia, and Visual Processing—Visual Processing and Functional Organization, Structure and Function of Central Visual Pathways

Publications

Munoz DP, Wurtz RH: Fixation cells in monkey superior colliculus. I. Characteristics of cell discharge. *J Neurophysiol* 70:559-575, 1993.

Munoz DP, Wurtz RH: Fixation cells in monkey superior colliculus. II. Reversible activation and deactivation. *J Neurophysiol* 70:576-589, 1993.

Munoz DP, Wurtz RH: Saccade-related activity in monkey superior colliculus. I. Characteristics of burst and buildup cells. *J Neurophysiol*, in press.

Wurtz RH, Munoz DP: Organization of saccade related neurons in monkey superior colliculus, in Buttner U, Brandt T, Fuchs A,

Wurtz RH, Munoz DP: Role of monkey superior colliculus in control of saccades and fixation, in Gazzaniga MS (ed): *The Cognitive Neurosciences.* Cambridge, MIT Press, 1994.

Zee D (eds): *Contemporary Ocular Motor and Vestibular Research: A Tribute to David A. Robinson.* International Meeting Eibsee, 1993. Stuttgart, New York, Georg Thieme Verlag and New York, Thieme Medical Publishers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00256-06 LSR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Information Processing by Visual System Neurons

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Lance M. Optican	Ph.D.	Chief, Neural Modeling Section	LSR, NEI
Others:	John W. McClurkin	Ph.D.	Staff Fellow	LSR, NEI
	Arthur V. Hays	B.A.	Electronics Engineer	LSR, NEI
	Brad J. Zoltick	M.A.	Computer Programmer	LSR, NEI
	Merk Na Chee-Orts	Ph.D.	Visiting Associate	LSR, NEI
	Marc H. Cohen	M.S.E.	Visiting Associate	LSR, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Neural Modeling Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.2	2.6	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our studies indicate that different visual areas in the brain may communicate via temporally modulated messages. We showed previously that neurons in different areas of the brain encode and transmit information about stationary, two-dimensional pictures that vary in form, brightness, and duration. We also showed that information about remembered visual features was also carried by a temporal code. Now we have extended those studies to show that neurons in visual cortex (areas V1, V2, and V4) carry information about the form and color of a stimulus in a temporally modulated code. Our results suggest that cortical neurons are able to convey information about many different features without confounding them. The mechanism for encoding these multiple messages uses temporal modulation to multiplex the different messages together on the neuron's response in a separable way.

It has been proposed that color and form information are divided into separate channels (*e.g.,* cytochrome oxidase blob and interblob regions) in the cortex. In a visual discrimination task studying the encoding of color and form information in cortical neurons, we showed that information about color and pattern rises over time in all neurons in cortical areas V1-V4. Such a result is not consistent with the idea that information about form and color is grouped into separate "channels" in cortex, but rather suggests that all neurons participate in visual processing, irrespective of the type of visual parameter involved.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

Perception and recognition of complex visual pictures depend on the normal function of interconnected brain regions extending from the retina through inferior temporal cortex. The properties of these regions are derived from the function of the single neurons within them. Thus, to understand how visual perception occurs, we must learn how information is encoded by the neurons in each stage of processing. If we could understand this neuronal code, it would become possible to distinguish between information related to the physical properties of a stimulus (*e.g.*, form, luminance, color, size) and information related to its behavioral significance (*e.g.*, leading to a reward). Individual neurons in all the visual areas studied thus far (retinal ganglion cell fibers; lateral geniculate nucleus neurons; pulvinar neurons; cortical neurons in visual areas V1, V2, and V4; and inferior temporal cortical neurons) encode and transmit information about stationary, two-dimensional pictures that vary in form, color, brightness, and duration. The neurons use a multidimensional temporal code to represent and transmit their stimulus-dependent messages. We have now shown that visual neurons convey complex messages about both a stimulus' physical parameters and its behavioral significance. Thus, using information theory, we can begin to explore how physical and behavioral components of a neuron's response contribute to higher visual cognitive functions such as perception, attention, and memory.

Major Findings

We have developed a new approach to studying single neurons in which they are treated as communication channels that transmit information about visual pictures in their responses. This approach has allowed us to apply methods from signal processing, statistics, systems analysis, and information theory to understand single neurons.

According to a commonly held view of neuronal function, the strength of a neuron's response represents how closely the stimulus matches the receptive field's characteristics (*e.g.*, orientation or color). Thus, if response strength were the only parameter a neuron could use to encode information, different stimulus features would be confounded by individual neurons. Using an informational analysis, we have shown that information about different stimulus parameters is not confounded but is carried across the different parts of the multidimensional neuronal code.

In recent experiments, responses of neurons in three visual cortical areas (V1, V2, and V4) were recorded from a monkey trained to choose one of three parafoveal stimuli based on whether their color or pattern matched that of a cue stimulus. These responses were modulated by the pattern and color of the stimulus on the receptive field and by the pattern or color of the preceding cue. In other experiments, stimuli consisted of colored bars that were isoluminant with the background or black or white bars that varied in size. Information about stimulus features developed continuously, but not uniformly, throughout the time-course of neuronal responses. Most of the information was encoded in the initial 50 to 60 milliseconds (msec) of the response. Some neurons also encoded a large amount of information in a second 50 msec interval, beginning 20 to 30 msecs after the first.

These results show that neurons in V1-V4 carry information about the color, pattern, contrast, and size of stimuli. Finally, the development of information over time in different areas suggests that temporally modulated waves of activity may form a code for visual information. In fact, the response to each stimulus could be represented as the product of two waveforms that were specific for the features paired in each stimulus (*e.g.*, color and pattern, color and orientation, contrast and orientation, or size and orientation). Feature-specific waveforms for each color, pattern, contrast, orientation, and size were isolated from the neuronal responses by a neural net. The product of these feature

waveforms predicted the neuronal responses to stimuli with feature combinations not used to train the neural net (*e.g.*, novel-colored patterns).

Code waveforms were often similar for all neurons within a cortical area. Waveforms encoding pattern were strikingly similar across all areas, irrespective of the behavioral task. These results suggest that neurons convey information about compound visual features by multiplexing feature-specific messages together. The invariance of the code waveforms suggests that information about a stimulus feature is represented similarly in all visual areas.

Significance to Biomedical Research and the Program of the Institute

This project studies how visual information is encoded and transmitted by neurons. Knowledge of these fundamental processes is important for understanding deficits of visual processing, such as occur in amblyopia, and for developing visual prosthetic devices to compensate for field defects or blindness.

Proposed Course

Discovering that the responses of visual system neurons are multidimensional led to the discovery that information about multiple stimulus features may not be confounded by single neurons, a result with important—even revolutionary—consequences. We now know that a substantial part of the temporal modulation arises after visual information has left the retina. Our latest results show that the neural code arises due to the influence of feedback.

Ever since we found evidence of a neural code, and saw a possible structure for it, we have been trying to delineate it. The properties of the code should give clues about the functions performed by the neurons. Now that we have shown that some of the temporal codes are invariant across cells, and even across areas, a new theory of visual information processing is required that will treat the visual system more as a concurrent processing system rather than as a hierarchical cascade of independent areas. Both these issues are being pursued.

Our findings suggest a completely new conceptional framework in which to investigate neuronal function. One presumed reason for the huge number of single neurons has been the necessity to unconfound stimulus features. However, we propose that the simultaneous messages about different features can be used as tags, so that the messages that arise in different processing regions of the visual system can be reunited into a unified percept. This would provide the mechanism to build a whole perception across many processing regions. With the use of new computational equipment, this hypothesis is being explored both experimentally and theoretically.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Visual Processing and Functional Organization, Structure and Function of Central Visual Pathways

Publications

McClurkin JW, Zarbock JA, Optican LM: Temporal codes for colors, patterns, and memories, in Peters A, Rockland KS (eds): *Cerebral Cortex, Vol. 10, Primary Visual Cortex of Primates.* New York, Plenum, 1994, pp 443-467.

McClurkin JW, Optican LM, Richmond BJ: Cortical feedback increases visual information transmitted by monkey parvocellular lateral geniculate nucleus neurons. *Vis Neurosci* 11:601-617, 1994.

Optican LM: Control of saccade trajectory by the superior colliculus, in Buttner U, Brandt T, Fuchs A, Zee D (eds): *Contemporary Ocular Motor and Vestibular Research. A Tribute to David A. Robinson.* International Meeting Eibsee, 1993. Stuttgart, New York, Georg Thieme Verlag and New York, Thieme Medical Publishers.

Ophthalmic Genetics and Clinical Services Branch

Report of the Chief
Ophthalmic Genetics and Clinical Services Branch

Muriel I. Kaiser-Kupfer, M.D.

The Ophthalmic Genetics and Clinical Services Branch (OGCSB) within the National Eye Institute (NEI) Intramural Research Program has been operational since February 1989. The branch is divided into four sections: Ophthalmic Genetics, Acting Chief Dr. Muriel I. Kaiser-Kupfer; Cataract and Corneal Diseases, Chief Dr. Manuel B. Datiles; Ophthalmic Pathology, Acting Chief Dr. W. Gerald Robison, Jr.; Clinical Services, Acting Chief Dr. Rafael C. Caruso.

The purpose of the OGCSB is to conduct clinical and laboratory research on gene expression and molecular interactions important to the eye and to apply clinically relevant research findings to the prevention, diagnosis, and treatment of diseases affecting the eye and the visual system. Such disorders include corneal and retinal diseases, cataract, and visual pathway abnormalities.

The OGCSB is responsible for the essential psychophysical and electrophysiological diagnostic tests of visual function required by all clinical intramural research programs of the National Institutes of Health (NIH). In addition, it processes ocular clinical biopsy and autopsy materials. The OGCSB differs from other NEI laboratories engaged in molecular investigations because its emphasis is on translating the appropriate research findings directly to the clinical setting. Thus, OGCSB is also a point of focus for the trans-NIH emphasis on research in genetics, more effectively aligning its organizational structure within the NEI's Intramural Research Program.

Since beginning its operation, the OGCSB has shown considerable growth and productivity.

Section on Cataract and Corneal Diseases

The Section on Cataract and Corneal Diseases continued to pursue research of the anterior segment, especially on the short-term and long-term effects of contact lens wear on the cornea. Analysis of the data may be helpful in understanding the dynamics of contact lens-cornea interaction, the risk to corneal tissues, and how systemic or local ocular disorders may increase the risk of wearing contact lenses. Corneal endothelial morphology is being studied by specular microscopy to compare the endothelial status in patients wearing different types of lenses. The development of automated computer analysis is under way to facilitate data analysis currently done by hand, which is time consuming and laborious.

This section has been particularly productive in studies using different systems to develop objective and subjective methods of monitoring and documenting opacities in the human lens. Objective systems being tested and developed include the Scheimpflug systems (Zeiss and Oxford) and the retroillumination camera (Neitz and Oxford). Subjective systems or methods such as the LOCS II grading system and the effects of cataracts on visual field, contrast sensitivity, and glare are also being studied and refined. These systems

are now being used to study the natural history of various cataracts such as presenile, senile or age-related, steroid induced, radiation, diabetic, retinitis pigmentosa, gyrate atrophy (GA), and neurofibromatosis 2 (NF2). Monitoring and documenting human cataract development is a crucial step toward the ultimate testing of several medications that might be helpful in preventing or reversing human cataracts. A large U.S. family has been ascertained, and the autosomal dominant cataracts with interocular variability has been identified. A second family has been identified in Hyderabad, India, and linkage has been established using micro markers.

Research in cataractogenesis has been hampered by the extreme scarcity of tissue and an abrupt shift in surgical technique from intracapsular (intact lens) to extracapsular (fragmented lens) extraction. Through the collaborative efforts of cataract surgeons and basic researchers, efforts have been under way to develop and modify techniques to study materials that become available at surgery and can be well documented clinically. We are presently performing careful documentation of the cataract in patients preoperatively using clinical aphotographic LOCS II grading and Zeiss Scheimpflug and Oxford retroillumination video photography and image analysis. Cataracts are extracted extracapsularly followed with implantation of an intraocular lens. Specimens obtained are examined histologically using light and electron microscopy and biochemically using two-dimensional gel electrophoresis (PHAST and LSB systems). Cataractous specimens are compared with normal tissues obtained from eye-bank eyes. Abnormal proteins are identified using immunoblotting techniques as well as by protein sequencing.

It has been demonstrated that with aging there is an acidic shift of proteins and an increased number of polypeptide species in the molecular weight range of the crystallins. It is of interest that these changes occur mainly in the lens nucleus and may be more for protection of lens proteins and preservation of lens clarity rather than leading to cataract formation. These studies are helping differentiate changes due to aging from changes occurring in cataract formation.

Investigators in this section have been in the forefront of recognizing the role of the neural crest in normal and abnormal development of the anterior segment. Studies continue on anterior chamber abnormalities and iridocorneal endothelial syndrome patients.

Section on Ophthalmic Genetics

Studies by the Section on Ophthalmic Genetics have emphasized retinal degeneration and ophthalmic involvement in systemic genetic diseases. This section has been a leader in studying GA of the choroid and retina. The accumulation of natural history data and the work on definition of the genetic abnormalities have been unique. Evidence for biochemical, clinical, and molecular heterogeneity continue to be confirmed. There appear to be many different single point mutations in the ornithine aminotransferase gene in GA patients. Dietary intervention studies using an arginine-deficient diet have been very promising, especially in young patients in whom a delay in the onset of pathologic changes has been demonstrated. GA is a condition that may be amenable to gene therapy; preliminary laboratory studies are under way. Usher's syndrome, congenital deafness, and retinitis pigmentosa patients are being studied using molecular techniques to map the gene and to identify the responsible mutation. Bietti crystalline retinopathy, a tapetoretinal degeneration with manifest corneal dystrophy is a rare condition apparently more common in Asians than in other ethnic groups. Linkage studies are under way to attempt to locate the gene responsible for this rare condition.

Foveal cone sensitivity (assessed by measurements of increment thresholds) and orientation (estimated with measurements of the Stiles-Crawford effect) were found to be abnormal in a group of patients with GA. These results suggest that foveal cones are

altered in their orientation and sensitivity before the encroachment on the foveal area by the atrophic lesions of GA. Genetic linkage studies are under way to pursue the gene(s) of this congenital cataract in this family.

Albinism in animals has been associated with an anatomic anomaly of the visual pathways characterized by an excessive crossing of the retino-geniculate fibers with two different modes of geniculo-cortical projection. In humans there is indirect evidence of the same anomaly demonstrated by asymmetry in visual evoked potentials (VEP) elicited by pattern reversal stimulation. Recent studies using appearing-disappearing patterns claim VEP asymmetry to be diagnostic and propose a uniform type of asymmetry. We used the same recording conditions to determine the diagnostic value of VEP in albinism and to attempt to correlate the VEP results with clinical features. This study shows that there are two different patterns of VEP asymmetry in albinism that may be explained by the differences in the reorganization of the geniculo-cortical pathway. VEP asymmetry is frequent but may not be constant in this condition. However, its value is decreased in some cases where the low amplitude of the responses makes the interpretation difficult. Furthermore, there is no correlation between the type of the asymmetry with any other feature of albinism.

Collaboration with the Interinstitute Genetics Program has continued with active participation by the Genetics Clinic. During the past year, approximately 200 individuals representing approximately 60 different disease categories have been seen. Because of the high frequency of ocular involvement in these cases, almost all of these patients were evaluated by the OGCSB staff.

NF2, otherwise known as bilateral acoustic neuroma, is inherited as an autosomal dominant disorder. Multiple members of several large pedigrees as well as a large number of unrelated families have been studied in collaboration with Dr. Dilys Parry, from the National Cancer Institute (NCI). An important original observation was the striking frequency (80 to 85 percent) of posterior capsular cataract in patients with NF2. In addition, 30 percent of patients have shown associated cortical cataracts. These findings are helpful in establishing a diagnosis of NF2 in at risk patients. The etiology of the cataract is unclear; however, it is interesting that the gene locus for bilateral acoustic neuromas is on chromosome 22 as is the gene for β-B-crystallin. Combined pigmented retinal harmartomas appear to be another ocular marker for some patients with severe NF2.

Finally, the results from the continuing double-masked control clinical trial of topically administered cysteamine to patients with nephropathic cystinosis are very exciting. After confirming the usefulness of 0.5 percent cysteamine eye drops in the young patients, we have expanded our study to include older patients, with similarly striking results. Particularly important is the fact that these patients have shown dramatic relief from their ocular symptoms with a decrease in crystals in the treated eye and a significant improvement of their quality of life.

Section on Clinical Services

The Section on Clinical Services has been very active in characterizing psychophysical and electrophysiological findings in patients with diseases that affect the eye and the visual system. Continued documentation by noninvasive techniques has shown that more and more refined and accurate classification of diseases is possible. Psychophysical and electrophysiological information is particularly helpful in understanding the pathogenesis of disease as well as being available for use as a marker in various treatment modalities.

Results of VEP studies have shown that a summation of two waveforms, which frequently show the same surface-positive polarity and are generated by stimulation of each hemifield, combine to generate the peaks of the full-field VEP.

Our results indicate that the sum of the asymmetrical contribution of either hemisphere and either eye are responsible for the symmetrical VEP elicited by binocular stimulation with a full-field stimulus. An asymmetrical full-field VEP may be seen in normal subjects and does not imply an abnormality in the visual pathways.

Studies of dark adaptation (DA) in patients with retinal dystrophies have indicated that a complete evaluation of DA should include, in addition to this measurement, the time constant of adaptation, that provides information about the rate at which this final threshold is reached. The time constant serves as a clinically relevant parameter in both the diagnosis of retinopathies and in the followup of individual patients over time.

Section on Ophthalmic Pathology

The Section on Ophthalmic Pathology has provided technical support services to investigators involved in clinical and basic research as well as to those performing routine pathology. Careful monitoring of the volume of material handled shows a steady increase in processing by the laboratory with excellent results. Considerable savings to the NEI have resulted from the elimination of costly contract services.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00187-11 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Effects of Corneal Contact Lenses on the Cornea

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
Others:	Gregory P. Kracher	O.D.	Expert	OGCSB, NEI
	Lessie M. McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Louella Lopez	M.D.	Visiting Associate	OGCSB, NEI
	Doretha Leftwood	B.A.	Computer Specialist	OGCSB, NEI
	Anup Mahurkar	B.S.	Visiting Associate	OGCSB, NEI

COOPERATING UNITS *(if any)*

Image Processing and Analysis Laboratory, Division of Computer Research and Technology, NIH (Mark Vivino, B.S., Staff Engineer)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.00	0.00	0.00

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

THIS PROJECT HAS BEEN TERMINATED

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00188-11 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Documentation and Monitoring of Opacities in the Human Lens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
Others:	Benjamin V. Magno	M.D.	Visiting Associate	OGCSB, NEI
	Anup Mahurkar	B.S.	Visiting Associate	OGCSB, NEI
	Doretha Leftwood	B.A.	Computer Specialist	OGCSB, NEI
	Joan Lee		Clinic Coordinator	OGCSB, NEI

COOPERATING UNITS *(if any)*

Image Processing and Analysis Laboratory, Division of Computer Research and Technology (DCRT), NIH (Benes Trus, Ph.D., Chief; Mark Vivino, B.S.); Biomedical, Engineering and Instrumentation Branch, DCRT, NIH (Michael Unser, Ph.D., Biomedical Engineer); Division of Epidemiology Clinical Trials, NEI (Valerie Friedlin, Ph.D. and Marvin Podgor, Ph.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.775	2.325	1.45

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project uses different systems to develop objective and subjective methods to monitor and document opacities in the human lens. We are actively recruiting patients with and without cataracts for reproducibility and followup studies on the objective systems—the Scheimpflug cameras (Zeiss and Oxford) and retroillumination (Neitz and Oxford) cameras. Our study of subjective systems or methods such as the LOCS II grading system and the effects of cataracts on visual perception, contrast sensitivity, and glare may be useful in identifying additional parameters for monitoring cataract presence, progression, or regression. In addition, we are modifying the systems to improve their accuracy and usefulness as well as developing outright improved systems and methods of documenting and monitoring progression of cataracts. We are now using these systems to study the natural history of various cataracts such as presenile, senile, or age-related, steroid-induced, radiation, diabetic, retinitis pigmentosa, gyrate atrophy, and neurofibromatosis 2 cataracts. This study will prepare the way for eventual clinical trials of anticataract drugs.

Genetic linkage studies under way are pursuing the gene(s) of congenital cataract.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Yvonne Duglas-Tabor	B.S.	Biologist, OGCS, NEI
Marvin Podgor	Ph.D.	Epidemiologist, DBE, NEI
Rita Hiller	M.S.	Epidemiologist, DBE, NEI
Robert Sperduto	M.D.	Chief, EB EB, NEI
Laura Wozencraft	M.S.	Genetic Counselor, OGCS, NEI
J. Fielding Hejtmancik	M.D., Ph.D.	Medical Officer, LMOD, NEI

Clinical Protocol Number

84-EI-0132

Objectives

The objective of this project is to formulate a means of documenting cataract formation and progression. This is an important step to take before undertaking clinical trials of drugs purported to prevent cataract and cataract progression. Family studies are involved in looking for the gene for congenital cataract via linkage studies.

Methods

Complete ophthalmologic examination, including contrast sensitivity, glare testing, and potential acuity testing, will be performed for each person in the study. Techniques used to measure and evaluate cataracts include Scheimpflug photography, retroillumination photography, specular microscopy, and laser light-scanning spectroscopy.

Major Findings

We have found that clinical grading of cataracts using the LOCS II system and photographic analysis of cataracts using the NEI Scheimpflug system can quantitatively detect the progression of age-related cataracts within one year. In addition, we found that in various types of cataracts, glare and contrast sensitivity testing showed abnormal results only in the severe or more advanced grades. The only exception was in posterior subcapsular cataracts, which showed an abnormality in contrast and glare sensitivity in the early stages based on LOCS II grading. In a study of pure nuclear cataracts, we found a significant correlation between lens nuclear density (using either LOCS II grading or Scheimpflug photography) and contrast sensitivity loss of intermediate and high spatial frequencies.

In our continued development of objective, semiautomated methods of detecting and following cataracts, we now are able to quickly perform densitometry of Scheimpflug nuclear cataract images and compare it with previous images to detect significant changes expressed in optical density units. For posterior subcapsular and cortical cataracts we have also developed a semiautomated method of quantitating the cataracts in mm^2 using retroillumination photographs.

Significance to Biomedical Research and the Program of the Institute

Monitoring and documenting human cataract progression is a crucial step toward the ultimate testing of several medications believed capable of preventing or reversing human cataracts. This step is also important in categorizing types of cataracts in various parts of the world and correlating them with the physical and genetic factors within specific geographic regions.

Subjective methods of determining visual function are also important to determine the handicap cataract patients have in coping with daily activities. In our studies none of the subjective methods could demonstrate subjective experiences in early cataracts; therefore, research is needed to develop more sensitive techniques.

Proposed Course

We will continue the study and development of subjective and objective methods of documenting and monitoring human cataracts. We

will pursue the improvement and automation of the present system of lens photography (*e.g.*, Scheimpflug, retroillumination, and laser-light spectroscopy) as well as exploration of possible applications of new technological advances. Appropriate population groups for study will be identified.

NEI Research Program

Lens and Cataracts—Epidemiology of Cataract

Publications

Datiles M: Progress in development and testing of anticataract medications. *Ophthalmology* 100:9A:70, 1993.

Datiles M, Lasa S, Podgor M, Hernandez-Galelia E, Magno B: Reproducibility of the NEI cortical cataract retroillumination system. *Curr Eye Res*, in press.

Datiles M, Magno B, Leftwood D, Friedlin V, Vivino M: Longitudinal study of age-related nuclear cataracts using the NEI Scheimpflug Imaging System. *Invest Ophthalmol Vis Sci* (Suppl) ARVO Abstract 34(4):943, 1993.

Kashima K, Trus B, Unser M, Datiles M, Edwards P, Sibug M: Aging studies on normal volunteer lenses using the Scheimpflug slit-lamp camera. *Invest Ophthalmol Vis Sci* 34:263-269, 1993.

Kashima K, Unser M, Datiles M, Trus B, Edwards P: Minimum views required to characterize cataracts when using the Scheimpflug camera. *Graef Arch Ophthalmol* 231:687-691, 1993.

Lasa S, Datiles M: Longitudinal study of age-related cortical cataracts using retroillumination photographs. *Invest Ophthalmol Vis Sci* 34(4):943, 1993.

Lasa S, Datiles M, Friedlin V: Potential vision tests in patients with cataracts. *Invest Ophthalmol Vis Sci* 35(4a):1962, 1994.

Lasa S, Podgor M, Datiles M, Magno B: Glare sensitivity in early cataracts. *Brit J Ophthalmol* 77:489-491, 1993.

Lopez L, Datiles M: Longitudinal study of age-related posterior subcapsular cataracts using retroillumination photographs. *Invest Ophthalmol Vis Sci* 34(4):943, 1993.

Lopez L, Datiles M, Podgor M, Vivino M, Mahurkar A, Lasa S: Reproducibiltiy study on the NEI retroillumination analysis system. *Invest Ophthalmol Vis Sci* 35(4a):1962, 1994.

Magno B, Datiles M, Lasa S: Cataract progression rates using the lens opacity classification system. *Invest Ophthalmol Vis Sci* 34:2138-2141, 1993.

Magno B, Friedlin V, Datiles M: Comparison of linear, multilinear and mask microdensitometric analysis of Scheimpflug images of the lens nucleus. *Curr Eye Res*, in press.

Magno B, Friedlin V, Datiles M: Reproducibility of the NEI Scheimpflug Imaging System. *Invest Ophthalmol Vis Sci* 35:3078-3084, 1994.

Vivino M, Chintalagiri S, Trus B, Datiles M: Development of a Scheimpflug slit lamp camera system for quantitative densitometric analysis. *Eye* 7:791-798, 1993.

Vivino M, Mahurkar A, Trus B, Lopez L, Datiles MB: Quantitative analysis of retroillumination images. *Invest Ophthalmol Vis Sci* 35(4a):1947, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00212-09 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Use of Human Lens Material for Determining Possible Causes of Cataracts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
Others:	Susan M. Lasa	M.D.	Visiting Associate	OGCSB, NEI
	Benjamin V. Magno	M.D.	Visiting Associate	OGCSB, NEI
	Yvonne Tabor	B.S.	Biological Technician	OGCSB, NEI
	Louella Lopez	M.D.	Visiting Associate	OGCSB, NEI
	Pushpa Sran	M.D.	Medical Officer	OGCSB, NEI

COOPERATING UNITS *(if any)*

Laboratory of Mechanisms of Ocular Diseases, NEI (Donita Garland, Ph.D.); Laboratory of Immunology, NEI (Miguel Burnier, Jr., M.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.05	1.75	1.3

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

There is an extreme scarcity of properly documented and classified human cataract material because of an abrupt shift of cataract surgical technique from intracapsular (intact lens) to extracapsular (fragmented lens) with the advent of the use of intraocular lens. We are exploring ways by which fragmented lens materials can be maximally used in cataract basic research through close collaboration with cataract surgeons and basic researchers and through modification of techniques by both groups.

We are now carefully documenting the cataracts preoperatively, using clinical and photographic LOCS II grading and Zeiss Scheimpflug and Oxford retroillumination video photography and image analysis. Cataracts are extracted extracapsularly with implantation of an intraocular lens. Specimens obtained are examined histologically, using light and electron microscopy, and biochemically, using two-dimensional gel electrophoresis (PHAST and LSB systems). Cataractous specimens are compared with normal tissues obtained from eye bank eyes. Abnormal proteins are identified using immunoblotting techniques as well as protein sequencing.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Samuel Zigler	Ph.D.	Head, Section on Cataracts, LMOD, NEI
Muriel I. Kaiser-Kupfer	M.D.	Chief, OGCS, NEI
J. Fielding Hejtmancik	M.D., Ph.D.	Medical Officer, LMOD, NEI

Clinical Protocol Number

84-EI-0194

Objectives

In light of the present and future scarcity of intact human cataracts for basic research, this study was designed to develop methods in which fragmented lens materials can be maximally used for biochemical and genetic research.

Methods

We examined patients who have different forms of cataracts and documented their cataracts as described in the project Documentation and Monitoring of Opacities in the Human Lens (Z01 EY 00188). After extracapsular cataract extraction, fragmented lens materials, including the anterior lens capsule, lens nucleus, and aspirated lens cortical material, undergo protein and biochemical analyses. Some of the specimens are frozen for future genetic studies. We also have undertaken studies to determine the visual results of current techniques of cataract surgery and how to modify and improve them further.

Major Findings

We have found that a successful collaboration requires a close professional relationship between the clinician-surgeon and the basic researcher. Although the two professionals have different immediate priorities (one being patient care, the other adequacy of tissue sample for laboratory studies), the ultimate goal of alleviating human suffering is the same.

The two-dimensional gel electrophoresis technique may be extremely useful in determining lens protein changes using very small amounts of tissue (300 mcg). Aging results in acidic shifting of proteins, an increased number of polypeptide species in the molecular weight range of the crystallins, and covalent cross-linking of crystallins—changes that need to be differentiated from changes occurring in cataract formation.

Recently, we found that in normal aging these age-related changes in lens proteins are confined to the nuclear region of the lens; the cortex remains the same. This means that these age-related changes are adaptations to maintain clarity of the nucleus and are not precataract. We also have found that lens material aspirated through the irrigator-aspirator or phakoemulsifier loses some crystallins. Optimum samples are those we obtain separately, thus avoiding oxidation.

Significance to Biomedical Research and the Program of the Institute

A severe setback is being dealt many cataract projects because of the lack of human cataract material available for basic research studies. Whereas the current technique, which involves fragmenting the cataract during extraction, is extremely successful and effective, there is no foreseeable change back to use of the intracapsular method (removal of the lens *in toto*). Hence, it is imperative to modify our basic research methodology to adapt to the use of fragmented lens materials to continue the various basic lens research projects that deal with human materials. We are already learning about possible differences between normal age-related changes in lens protein and precataract. This knowledge is crucial for the eventual understanding of the cause of cataracts.

Proposed Course

We will continue to pursue the development of the use of fragmented lens material in basic

research experiments. Using two-dimensional gel electrophoresis, we will study ways in which surgeons may modify their surgical techniques without compromising patient care while providing scientists with critical lens tissue for basic research. In addition, we will investigate ways in which scientists can work with surgeons in handling lens materials to maximize the quality of specimens for basic research.

NEI Research Program

Lens and Cataract—Pathogenesis of Cataract

Publications

Datiles M: Progress in development and testing of anticataract medications. *Ophthalmology* (suppl) 100(9A):70, 1993.

Garland D, Datiles M, Zigler J, Duglas-Tabor Y: Post translational modification of human crystalline. *Invest Ophthalmol Vis Sci* 35(a):1904, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00281-02 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Addendum to Use of Human Lens Material for Determining Possible Causes of Cataracts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	J. Fielding Hejtmancik	M.D., Ph.D.	Medical Officer	LMOD, NEI
	Laura A. Wozencraft	M.S.	Genetic Counselor	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.325	1.325	0.0

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects   [ ] (b) Human tissues   [ ] (c) Neither

[X] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Although the etiologies of some secondary cataracts are becoming better understood and certain animal models show promise for elucidating the relationships between lens crystalline and hereditary cataract, little is known about the causes of congenital cataracts in humans. To date, the classification of different congenital cataracts has been cumbersome and imperfect. A better understanding of cataractogenesis will come through an understanding of the molecular components of the lens of the eye and the ways in which lesions of these components are manifested, structurally and functionally, as opacity of the lens. Animal studies have suggested that alterations in lens crystallins can cause hereditary cataracts, making them reasonable candidate genes for causing hereditary cataracts in humans. In addition, it is apparent that hereditary lesions that mimic or contribute additively to environmental stress known to cause cataracts might be candidate genes for causing hereditary cataracts. The work in this project is designed to concentrate specifically on congenital and hereditary cataracts and to take full advantage of molecular technology developed for linkage analysis. Studies performed on informative families will include collecting blood specimens from available family members and, when possible, analyzing lens material from patients who undergo cataract surgery.

Project Description

Clinical Protocol Number

84 EI-0194A

Objectives

The objective of this study is to localize, identify, and study by linkage analysis and other molecular genetic techniques the genes that cause hereditary cataracts.

Methods

Linkage analysis (gene mapping) will be carried out by following the inheritance of genetic markers in families with hereditary cataracts. Blood specimens will be obtained and analyzed for gene marker linkage analysis from informative families.

Major Findings

At present, the process is under way to accumulate families with congenital cataracts; a large family with known autosomal dominant congenital cataract has been analyzed. Of note, it has shown for the first time evidence of intraocular phenotypic heterogeneity in a family with autosomal dominant congenital cataract. Studies for markers for gene analysis are under way.

Significance to Biomedical Research and the Program of the Institute

By studying patients with congenital inherited cataract, it may be possible to find the specific gene that is responsible for the development for congenital cataracts.

Proposed Course

Additional families will be recruited that have congenital cataract, and linkage analysis will be performed on these families.

NEI Research Program

Lens and Cataract—Pathogenesis of Cataract

Publications

Hejtmancik JF, Kaiser MI, Piatigorsky J: Molecular biology and inherited disorders of the eye lens, in Scriver CR, Beaudet Al, Sly WS, Valle D, (eds): *Metabolic Basics of Inherited Disease.* New York, McGraw-Hill Inc., in press.

Scott MH, Hejtmancik JF, Wozencraft LA, Reuter LM, Parks MM, and Kaiser-Kupfer MI: Autosomal dominant congenital cataract: Interocular phenotypic heterogeneity. *Ophthalmology* 101:866-871, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00084-16 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Anterior Chamber Anomalies Associated With Glaucoma or Ocular Hypertension

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Carl Kupfer	M.D.	Director	NEI
Others:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
	Lessie M. McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
	Susan M. Lasa	M.D.	Visiting Associate	OGCSB, NEI
	Benjamin V. Magno	M.D.	Visiting Associate	OGCSB, NEI
	Louella Lopez	M.D.	Visiting Associate	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.85	.75	0.10

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither
[ ] (a1) Minors
[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recent embryological research has indicated the role of the neural crest in contributing to all connective tissues anterior to the lens epithelium. Therefore, the group of developmental anomalies of the anterior chamber with glaucoma or ocular hypertension is being reviewed.

PHS 6040 (Rev. 5/92)

Project Description

Clinical Protocol Number

77-EI-0119

Objectives

The objective of this study is to determine whether congenital or developmental anomalies of the anterior chamber are related to faulty migration or terminal differentiation of neural crest tissue.

Methods

Patients of all ages with congenital or developmental anomalies of the anterior chamber are being examined to determine involvement of cornea, trabecular meshwork, iris stroma, lens, and ciliary body. When intractable glaucoma cannot be controlled with medication, surgery is performed; the specimens are examined histologically. When the lenses become cataractous, cataract extractions are performed; the lens epithelium is grown in tissue culture. When the cornea is opaque and corneal transplantation is indicated, that procedure is performed; the corneal specimen is examined histologically.

Major Findings

It appears that in this group of anomalies of anterior chamber development, there are pathological changes in one or several tissues derived from neural crest. These changes include corneal stroma, corneal endothelium, anterior iris stroma, Descemet's membrane, and trabecular meshwork endothelium.

We have recently performed trabeculectomies on patients with the irido-corneal-endothelial syndrome. Histopathologically, we found the presence of a membrane covering the trabecular meshwork. That membrane may have caused the peripheral anterior synechias and glaucoma.

Significance to Biomedical Research and the Program of the Institute

A better understanding of the pathogenesis of this glaucoma may help by improving diagnosis and treatment. The presence of this membrane may explain glaucoma's progressive nature and suggest possible surgical or laser treatments as a way to control or prevent the progression of the disease.

Proposed Course

Patients with other anomalies of the anterior chamber, including congenital cataracts, will be examined for abnormalities in tissue derived from neural crests. We will continue to study cases of congenital corneal disorders to uncover the cause and to determine treatment choices for these cases.

NEI Research Program

Glaucoma—Basic Science Research, Pathobiology and Morphology

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00011-20 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pigment Dispersion With and Without Glaucoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Lessie M. McCain	R.N.	Nurse Specialist	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.20	0.1	0.1

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[X] (a1) Minors

[X] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to determine the risks of patients with pigment dispersion syndrome for glaucoma. Comparisons of patients with and without glaucoma are made on the basis of diagnostic tests, genetic screening, and aqueous humor dynamics. The data acquired may enable determination of pigment dispersion syndrome patients' risk of developing glaucoma as well as adding to the understanding of the pathology of the disease.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Marvin Podgor Ph.D. Statistician, BEP, NEI

Clinical Protocol Number

76 EI-0189

Objectives

This project was designed (1) to compare patients with and without glaucoma who have pigment dispersion by documenting and following the clinical features and courses of disease and by evaluating performance on a variety of diagnostic tests, (2) to determine the presence of abnormal aqueous humor dynamics in glaucoma and nonglaucoma patients with pigmentary dispersion, and (3) to compare the association of pigment dispersion, with and without glaucoma, with possible genetic markers.

Methods

A complete evaluation included the following: complete family history with detailed pedigree; best-corrected visual acuity with manifest refraction; slit-lamp biomicroscopy; visual field examination (Goldmann I_2e and I_4e); applanation Goldmann tension; photography of iris color, iris transillumination, and Krukenberg spindle; A-scan, anterior chamber depth, and anterior chamber volume measurements; goniophotography; static perimetry; fasting blood sugar when indicated; dilated ophthalmoscopic examination (using two and one-half percent phenylephrine and one percent cyclogel); and stereophotographs of the optic nervehead.

Major Findings

One hundred forty-two patients were classified into three groups: (1) pigment dispersion syndrome (PDS) without abnormal ocular pressure, (2) PDS with ocular hypertension, and (3) PDS with glaucoma. Analysis of baseline characteristics with respect to anatomical and physiological parameters has yielded the following conclusions:

(1) It appears that the majority of patients recruited have PDS with a benign course; they do not develop ocular hypertension or glaucoma.

(2) PDS may be familial and can show a dominant inheritance pattern. Consequently, family members of PDS patients should be alerted and appropriately screened.

(3) Analyses of graded iris transillumination, the amount of pigment deposited on the trabecular meshwork, and the anterior chamber depth have demonstrated no significant differences among the three categories of PDS. Thus, pigment deposited in the angle may be only a secondary factor adversely affecting an already compromised outflow facility that is primarily a result of the presence of open-angle glaucoma.

(4) It also appears that those patients who develop ocular hypertension and demonstrate early field changes can be managed medically by control of intraocular pressure and reversal of early field loss. Patients who develop glaucoma do not appear to be more difficult to treat than patients with open-angle glaucoma.

(5) The phenomenon of unilateral or asymmetric pigment dispersion syndrome with little difference between the measurements of the two eyes is being investigated in followup studies.

(6) In our series, retinal detachment does not appear to occur with any greater frequency than with high myopia. A history of asymptomatic and nonprogressive peripheral retinal holes was noted in two patients.

(7) The data from this study have been computerized, and an indepth analysis is under way.

Significance to Biomedical Research and the Program of the Institute

These results may facilitate determination of the risk of the development of glaucoma for patients with pigment dispersion. Specifically, it may be possible to identify which features have predictive value in forecasting which PDS patients will develop visual field defects. In addition, the data will aid investigation of the relationship of "pigmentary" glaucoma to the known characteristics of open-angle glaucoma.

Proposed Course

This project will be continued for three more years to obtain additional data on the patients enrolled in the study.

NEI Research Program

Glaucoma—Epidemiology and Clinical Research, Treatment

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00060-16 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visual Function and Ocular Pigmentation in Albinism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Lessie M. McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
	Leanne Reuter	B.S.	Research Ophthalmic Technician	OGCGB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.31	0.26	0.05

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[X] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with hypomelanotic disorders such as ocular albinism, oculocutaneous albinism, Chediak-Higashi disease, Hermansky-Pudlak syndrome, and iris transillumination defects are being recruited to determine visual function with these conditions and to evaluate the changes in visual function over time. Family members are evaluated to attempt to determine factors that may identify the heterozygous state.

PHS 6040 (Rev. 5/92)

Project Description

Clinical Protocol Number

76 EI-0207

Objectives

The objectives of this study are (1) to relate the level of visual function to the amount of ocular pigmentation, especially iris and retinal pigmentation; (2) to correlate the amount of nystagmus with visual acuity and iris pigmentation; (3) to determine whether ocular pigmentation, visual acuity, and nystagmus change with age; (4) to determine whether abnormalities of crossing of the optic nerve fibers can be correlated to the lack of pigmentation and to determine if previous reports in abnormalities of crossing can be confirmed.

Methods

For each patient, a complete family history with detailed pedigree is compiled and the following procedures are performed: best-corrected visual acuity near and at distance with refraction; slit-lamp examination; psychophysical testing, including D-15 and Munsell 100 hue and rod and cone thresholds; dilated ophthalmoscopic examination; photography to document hair color, eye color, iris transillumination, and the status of the disc and macula; visual evoked response testing; and, in selected patients, contrast sensitivity measurements. Information on family members is collected by examination of best corrected visual acuity, slit-lamp examination of iris, photography of iris transillumination, and fundus examination when vision is not corrected to 20/20.

Major Findings

(1) Examination of 84 patients and family members indicated that transillumination of the iris may be seen in the absence of recognized albinism. The pattern, which appears to be punctate, may be present in a diffuse manner or limited to the six o'clock sector. The finding is not associated with nystagmus.

(2) These patients presented with marked iris transillumination, reduced pigmentation of the fundus, and no nystagmus, but they had decreased visual acuity, which has improved in conjunction with an increase in the pigmentation of the fundae.

(3) Visually evoked responses were normal in some patients, but in a subset of albinos there was evidence of nonuniform pattern of asymmetry in the results of the miswiring of the visual pathways. Low amplitude of the visual evoked potentials recorded in a consecutive series of patients shows the difficulties of the study of this phenomenon in a clinical setting.

Significance to Biomedical Research and the Program of the Institute

Determination of whether the development of the fovea is abnormal in albinism, whether this abnormal foveal development is the cause of the decreased visual acuity in albinism, or, alternatively, whether decreased visual acuity is secondary to hypopigmentation and the resultant light scatter and glare may be possible. Collection of these data also will facilitate ascertainment of whether visual acuity improves with age and whether this correlates with changes in pigmentation.

In addition, studies are being conducted to verify the reported findings of abnormalities of the crossing fibers, as measured by visually evoked responses, contrast sensitivity, degree of nystagmus, and amount of pigmentation.

Proposed Course

This project will be continued for five more years to obtain additional data.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Bouzas EA, Caruso RC, Drews-Bankiewicz MA, Kaiser-Kupfer MI: Evoked potential analysis of visual pathways in human albinism. *Ophthalmology* 101:309-314, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00083-17 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gyrate Atrophy of the Choroid and Retina and Other Retinal Degenerations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Fumino Iwata	M.D.	Special Volunteer	OGCSB, NEI
	Lessie M. McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
	Pushpa K. Sran	M.D.	Medical Officer	OGCSB, NEI
	Doris J. Collie	A.A.	Ophthalmic Technician	OGCSB, NEI
	John B. Christoforidis	M.D.		IRTA, OGCSB, NEI

COOPERATING UNITS *(if any)*

Howard Hughes Medical Institute, Laboratory and Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, MD (David L. Valle, M.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	0.8	0.4

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[x] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with gyrate atrophy of the choroid and retina are examined systematically to confirm the diagnosis. Skin fibroblasts from affected patients and family members are grown in tissue culture and assayed for ornithine aminotransferase activity. The results are evaluated for correlation with the presence of homozygosity or heterozygosity for the disease trait. Each patient is given a trial of pyridoxine to see whether serum concentration of ornithine can be reduced; if so, the patient is classified as a "responder," and treatment with pyridoxine is continued. Nonresponder and responder patients are then placed on a low-arginine, low-protein diet with supplemental amino acids and observed for arrest or improvement of the disease. If patients are not considered eligible for the diet, or if they appear unable to comply with the dietary regimen, we follow them to record the natural progression of the condition. Patients with other forms of retinal degeneration such as retinitis pigmentosa, fundus flavimaculatus, juvenile retinoschisis, and Bietti's crystalline dystrophy, are also examined. The courses of their diseases are compared with those of gyrate atrophy patients.

PHS 6040 (Rev. 5/92)

Project Description

Additional Personnel

Laura Wozencraft	M.S.	Genetic Counselor, OGCSB, NEI
J. Fielding Hejtmancik	M.D., Ph.D.	Medical Officer, LMOD, NEI

Clinical Protocol Number

78 EI-0001

Objectives

This project is being conducted to: (1) determine the biochemical processes responsible for the elevated plasma ornithine and the chorioretinal lesions that occur in gyrate atrophy (GA) of the choroid and retina; (2) determine which patients respond to pyridoxine treatment with a decrease in plasma ornithine concentration; (3) determine whether treating "responders" with pyridoxine and nonresponders with an arginine-deficient diet will arrest the progress of chorioretinal atrophy; (4) study the natural history of this condition when intervention is not undertaken and to determine the degree of heterogeneity; (5) define the molecular mutations and compare the molecular defect with the clinical features of the disease; and (6) characterize and follow the progression of lens opacities, obtaining lens specimens at the time of cataract extraction for protein analysis; and (7) evaluate patients for abnormal thyroid function.

Methods

Patients suspected of having GA of the choroid and retina are examined according to a standard set of procedures to confirm the diagnosis. Plasma ornithine concentration is measured periodically. Punch biopsies of the skin are grown in tissue culture; their ornithine aminotransferase activity is measured, and patient molecular defect is characterized. Complete evaluation of ocular function in these patients includes best corrected visual acuity, Goldmann visual fields, color vision, cone thresholds, dark adaptation, electroretinogram (ERG), foveal ERG, electrooculogram, contrast sensitivity, and Stiles Crawford effect.

Major Findings

GA, a rare autosomal recessive disorder, is associated with hyperornithinemia, overflow ornithinuria, and a deficiency of activity of the mitochondrial enzyme ornithine-δ-aminotransferase (OAT). Although rare, the condition has been described worldwide in all races. Forty-seven patients have been recruited and evaluated in this study. The patients' ethnic origins vary and include African American, Asian, Indian, English, Finnish, German, Israeli, Lebanese, Polish, Portuguese, Scottish, Turkish, and Welsh.

This study included 23 females and 24 males, ranging in age from 2.5 to 65 years, with 12 children younger than 12 years at the time of recruitment. Observations of these patients have enabled documentation of both clinical evidence and laboratory heterogeneity.

Analysis of the mutation that causes GA of the choroid and retina has been undertaken by Drs. David Valle and Grant Mitchell and colleagues, from The Johns Hopkins University. They have analyzed probands from 72 GA pedigrees. No gross structural alterations of the OAT gene have been detected; 85 percent of the probands express nearly normal amounts of normal-sized OAT messenger ribonucleic acid (mRNA). The remainder express little or no OAT mRNA (n = five) or an mRNA with an altered size (n = two). Western blot studies showed the OAT antigen was absent in 67 percent of the mRNA+ mutants and in all of the mRNA-mutants. A total of 14 mutations have been delineated at the molecular level: 10 missense mutations (M1I, R180T, L402P, C93F, Y55H, R154L, A270P, R271KL, G375V, and P417L/L437F), a single nucleotide deletion at complementary deoxyribonucleic (cDNA) position +159 (H53fs), an interesting in-frame three-nucleotide deletion of Ala-184 (A185F0), and a nonsense mutation at a CpG dinucleotide (R396ter).

The functional consequences of several mutations have been examined by substituting the mutations into otherwise wild-type OAT cDNA in the expression vector P91023b and transfecting the recombinant constructs into CHO-K1 cells that lack endogenous OAT mRNA or protein. Three (R180T, L402P, A184D0) have been shown to encode a CRM+, enzymatically inactive protein while M1I—as expected for an initiation codon alteration—has a CRM-phenotype. Studies are under way to correlate mutational heterogeneity with clinical and biochemical heterogeneity.

The earliest clinical and electrophysiologic features were documented in the three youngest patients (ages two, two and one-half, and three years). When significant reduction of rod and cone function is seen by electroretinographic studies, the minimal evidence of clinical retinal changes is noteworthy.

Clinical and biochemical evidence of genetic heterogeneity is present in these patients. Fewer than 10 percent of patients have been reported to have a 30 to 50 percent decrease in plasma ornithine following treatment with vitamin B_6. Only one of our patients showed an *in vivo* response to this treatment. Comparisons of sibships reveal that there is a greater degree of interfamilial variability than intrafamilial variability.

Whereas arginine is the precursor of ornithine in the metabolic pathway of ornithine metabolism, we have undertaken a dietary intervention study limiting arginine. Among 25 patients placed on a low protein (low-arginine) diet, all sustained significant reduction of ornithine during hospitalization; however, the diet was discontinued in four Finnish patients following their discharge because of poor compliance and in seven other patients because of a variety of factors. Among 12 patients remaining on the diet, four have excellent control; four, fair control; four, erratic control. Ophthalmologic evaluations are performed on all patients every six to 12 months, travel permitting.

In two patients with the best biochemical control for the longest time (16 and 15.3 years), there was evidence of improved visual function. One patient after being on the diet for 14 months showed improved dark adaption, average ERG, and color vision. This improvement was sustained for 30 months, then the ERG amplitude showed a small but definite reduction. The second patient with lowered plasma ornithine levels, who had been on the diet for 11 years, showed progressive improvement in visual field and color vision and has since remained stable. A third patient, despite fair control, was stable for 36 months but has deteriorated for the past 18 months. It should be noted that she was the oldest patient and had the most advanced disease at the outset. Other patients followed for various periods currently appear stable. Of particular interest are the children who were ages two and one-half to nine years at the outset of diet. Results indicate that as a result of dietary intervention, the course of the disease in the younger child of each sibship has been improved compared with that of the older sibling.

All but one patient older than age 11 years have had progressive cataracts in the posterior capsule. These cataracts present a uniform histologic picture and can be identified by their characteristic pattern in image analysis.

Significance to Biomedical Research and the Program of the Institute

GA of the choroid and retina is the first of the genetically determined isolated severe retinal degenerations for which a specific biochemical marker and a concomitant enzyme defect have been demonstrated. This study of treatment efficacy for this blinding eye disease will serve as a model for the investigation of other genetically determined retinal degenerations. Study of the two young patients is the best opportunity for the evaluation of diet control. This disease is a likely candidate for future studies to begin gene therapy.

Proposed Course

This project will be continued for three more years to assess further the importance of reduced ornithine in halting chorioretinal degeneration.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Kaiser-Kupfer MI, Chan C-C, Markello TC, Crawford MA, Caruso RC, Csaky KG, Guo J, Gahl WA: Natural history and biochemical and clinical pathologic correlations in Bietti's crystalline dystrophy. *Am J Ophthalmol,* in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00163-12 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

NIH Interinstitute Genetics Program: The Genetics Clinic

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Fumino Iwata	M.D.	Special Volunteer	OGCSB, NEI
	Lessie M. McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Anren Li	M.D.	Visiting Associate	OGCSB, NEI
	Laura A. Wozencraft	M.S.	Genetic Counselor	OGCSB, NEI

COOPERATING UNITS *(if any)*

Interinstitute Medical Genetics Program, NIH

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.85	0.35	0.5

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[x] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Interinstitute Genetics Program and the Genetics Clinic supported by the Clinical Center offer a multidisciplinary approach to patients with genetic disease (Z01 CP 05139-06 CEB). Involved in the program are researchers from all Institutes. Patients evaluated in the clinic represent a broad spectrum of genetic diseases. During the past year, the approximately 200 persons seen represented about 60 distinct disease categories. Due to the high frequency of ocular involvement in many of the cases, almost all the patients were evaluated by Clinical Branch staff or were discussed in consultation. The clinic serves as a source of interesting case material concerning patients with inherited or developmental abnormalities of the visual system.

PHS 6040 (Rev. 5/92)

Project Description

Clinical Protocol Number

Interinstitute Medical Genetics Program

Objectives

The objectives of this program are to (1) evaluate patients with ocular abnormalities associated with genetic disease in the context of a multidisciplinary approach to the patient; (2) provide genetic counseling to patients at risk for inherited ocular disease; (3) recommend and advise appropriate evaluation for the ocular problem; (4) provide training in the diagnosis, counseling, and treatment of individuals with or at risk for genetic disease as well as in the research approach to genetic disease.

Methods

Referred patients are examined, and the appropriate diagnostic ophthalmologic workup is recommended.

Major Findings

(1) Iris nodules were commonly seen in the classic cases of neurofibromatosis (NF)1 and less frequently seen in patients with less well-defined disease. They were seen rarely in patients with bilateral acoustic neuroma or NF2. Interestingly, in a series of 14 consecutive patients with Cushing's disease, two patients (14 percent) had typical, unilateral lisch nodules. To our knowledge the association of NF1 on lisch nodules with Cushing's disease has not been described. The association of Cushing's disease and lisch nodules reported may represent a mild form of multiple endocrine neoplasia of the mixed type. It is possible that a common underlying mechanism leads to the overgrowth of melanocytes in the iris and corticotropin in the pituitary. Eighty percent of the patients with NF2 showed increased frequency of posterior capsular cataracts; this information serves as an excellent marker. A new finding is the association of peripheral cortical cataracts in 37.8 percent of NF2 patients. In a group of severely affected NF2 patients, it appears that combined pigment epithelial and retinal hamartomas are also an ocular marker for NF2. In fact, there may be a predication for the macula in some cases.

(2) Serious ocular complications were observed in 13 long-term postrenal transplantation nephropathic cystinosis patients. These complications included decreased visual acuity and visual function, as measured by psychophysical and electrodiagnostic tests, band keratopathy, and posterior synechia. Corneal transplantation may be necessary in cases with debilitating symptoms from recurrent erosion after all other treatment modalities have failed. In two such patients, the corneal grafts have remained clear for as long as seven years.

(3) Ophthalmic studies performed in a population of patients with endogenous Cushing's syndrome revealed that posterior subcapsular cataracts were an infrequent phenomenon compared with exogenous Cushing's syndrome. Central serous chorioretinopathy, although an uncommon finding, was seen in three of 60 patients (five percent) suggesting that glucocorticoids may play a role in the development of the disease.

Significance to Biomedical Research and the Program of the Institute

Genetic and developmental anomalies of the eye are a major cause of blindness and visual disability, and they are responsible for about 35 percent of the cases of blindness in developed nations. Involvement with the Interinstitute Medical Genetics Program affords a systematic approach to studying these and other conditions associated with genetic diseases.

Proposed Course

The project is in a growth phase and will be expanding in future years.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Bouzas EA, Mastorakos G, Friedmann T, Scott MH, Chrousos GP, Kaiser-Kupfer MI: Posterior subcapsular cataracts in endogenous Cushing syndrome: An uncommon manifestation. *Invest Ophthalmol Vis Sci* 34:3497-3500, 1993.

Bouzas EA, Scott MH, Mastorakos G, Chrousos GP, Kaiser-Kupfer MI: Central serous chorioretinopathy in endogenous hypercortisolism. *Arch Ophthalmol* 111:1229-1233, 1993.

Iwata F, Kaiser-Kupfer MI: Ocular manifestations of metabolic disorders. *Curr Opinion Ophthalmol*, in press.

Parry DM, Eldridge R, Kaiser-Kupfer MI, Bouzas EA, Pikus A, Patronas N: Neurofibromatosis 2 (NF2): Clinical characteristics of 63 affected individuals and clinical evidence for heterogeneity. *Am J Med Genet*, in press.

Theodoropoulos DS, Krasnewich D, Kaiser-Kupfer MI, Gahl W: Classical nephropathic cystinosis as an adult disease. *JAMA* 270:2200-2204, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00282-02 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Usher Syndrome—Clinical and Molecular Studies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	J. Fielding Hejtmancik	M.D., Ph.D.	Medical Officer	LMOD, NEI
	Fumino Iwata	M.D.	Special Volunteer	OGCSB, NEI
	Rafael C. Caruso	M.D.	Visiting Scientist	OGCSB, NEI
	Laura A. Wozencraft	M.S.	Genetic Counselor	OGCSB, NEI
	Anita Pikus	M.A.	Chief, Audiology Unit	HS/NOB, NIDCD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[x] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to document the clinical features of Usher syndrome and to refine the localization and eventually to isolate the genes causing this disease.

PHS 6040 (Rev. 5/92)

Project Description

Clinical Protocol Number

93 EI-0161

Objectives

The objectives of this study are to classify as accurately as possible the patient's clinical features by careful audiologic, vestibular, psychophysical, and electrodiagnostic testing and to correlate these with genetic mutations identified through linkage studies eventually to the genes (genetic mutations) as they become identified.

Methods

Included in the evaluation will be audiometric, vestibular, ophthalmologic, and electrophysiologic and electrodiagnostic testing. These clinical findings will help to classify the features of the different types of Usher's syndrome as well as to correlate the phenotypic features with the genetic mutation. To identify the genetic mutation, we will study informative families, collecting blood specimens from all available family members for studies using molecular technology developed for linkage analysis. In those cases where there are no other affected family members, blood specimens will be obtained to study specific gene mutations when the specific gene or genes for Usher's syndrome are identifiable.

Major Findings

Recruitment in this project has begun, and 50 patients have been recruited. Patients are being evaluated, and their blood specimens are being collected. Transformed lymphoblastic lines are being maintained in the laboratory. Linkage analysis on these families has not yet begun.

Significance to Biomedical Research and the Program of the Institute

By molecular studies of patients with Usher's syndrome, mutations may be correlated with clinical findings and genes responsible for Usher's syndrome may be defined, leading to more accurate counseling and diagnosis and the possibility of genetic therapy at some point.

Proposed Course

Patients will continue to be recruited into the study.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00283-02 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A Double-Masked Controlled Randomized Clinical Trial of Topical Cysteamine [II]

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Muriel I. Kaiser-Kupfer M.D. Chief OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[X] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

THIS PROJECT HAS BEEN TERMINATED

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 EY 00211-09 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A Double-Masked Controlled Randomized Clinical Trial of Topical Cysteamine [I]

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Lessie M. McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
	Fumino Iwata	M.D.	Special Volunteer	OGCSB, NEI
	Anren Li	M.D.	Visiting Associate	OGCSB, NEI
	Laura A. Wozencraft	M.S.	Genetics Counselor	OGCSB, NEI

COOPERATING UNITS *(if any)*

Human Genetics Branch, National Institute of Child Health and Human Development, NIH, Bethesda, MD (William Gahl, M.D., Ph.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.70	0.45	0.25

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[X] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Nephropathic cystinosis is an autosomal, recessively inherited storage disease in which nonprotein cystine accumulates within cellular lysosomes due to a defect in lysosomal cystine transport. Ocular manifestations include photophobia; crystal deposits in the cornea, conjunctiva, and iris; and depigmentation of the retina.

Ten years ago, cysteamine, a free thiol that depletes cystine from cells, was introduced in the therapy of cystinotic patients. Although patients had improved growth and stabilized renal function, there was no noticeable effect on the accumulation of corneal crystals. Recent studies showed that corneal cells in tissue culture are readily depleted of cystine by the introduction of cysteamine, making feasible the use of topical ophthalmic cysteamine to circumvent the humoral route. After appropriate animal studies to test for complications revealed none, we began a double-masked clinical trial to test the efficacy of topical cysteamine (0.1 percent and 0.5 percent) in humans.

To date, in 15 of 29 young patients the code was successfully broken; of the 14 remaining, two died, five discontinued medication, and 7 are still in the trial with poor compliance and have not been seen for followup. Because of the success in the younger patients, this study was expanded to include older patients, three to 31 years of age. The findings have been most exciting: Twenty-four patients have shown a significant decrease in crystals in treated eyes as well as improvements in comfort, i.e., relief of pain and photophobia. This study has resulted in significantly improved quality of life for the successfully treated patients. Because of the success of this clinical trial and evidence from the cysteamine-benzalkonium trial (Protocol Number 93 EI-0230), the Food and Drug Administration has requested that all patients in this protocol be switched to cysteamine plus benzalkonium and receive medication in both eyes. Each patient then will be judged by a comparison with his or her own natural history.

Project Description

Additional Personnel

Ernest M. Kuehl	Chief, Photography Section, OGCSB, NEI

Clinical Protocol Number

86 EI-0062

Objectives

The purpose of this project is to test the efficacy of topical cysteamine in patients with nephropathic cystinosis.

Methods

Slit-lamp examination and photography of the cornea are performed by a masked observer to determine whether there is a difference in the quantity of crystals seen in the cornea.

Major Findings

Topical cysteamine eyedrops (0.5 percent) are well tolerated. The crystal accumulation is reversible in very young patients who do not have crystals packing the cornea as well as in older patients in whom the crystals pack the cornea.

Significance to Biomedical Research and the Program of the Institute

The continued accumulation of crystals in the cornea appears to lead to increasing discomfort in cystinosis patients who develop severe photophobia with recurrent corneal erosions. Topical cysteamine treatment, which has been found to halt the process, has led to an improvement in the quality of life of these patients.

Proposed Course

This study will be replaced by a study in which the crystal accumulation will be compared with the natural history of the condition.

NEI Research Program

Corneal Diseases—Tears and Ocular Surface Epithelium

Publications

Theodoropoulos DS, Krasnewich D, Kaiser-Kupfer MI, Gahl W: Classical nephropathic cystinosis as an adult disease. *JAMA* 270:2200-2204, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00284-02 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characteristics of Macular Scotomas in Patients With Primary Monofixation Syndrome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Mark H. Scott	M.D.	Senior Staff Fellow	OGCSB, NEI
Others:	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI

COOPERATING UNITS *(if any)*

Marshall M. Parks, M.D. (Private Practice), Washington, DC

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[X] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

THIS PROJECT HAS BEEN TERMINATED

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00123-14 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Clinical Psychophysics of the Visual System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
Others:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
	Doris J. Collie	C.O.M.T.	Research Ophthalmic Technician	OGCSB, NEI
	Leanne M. Reuter	C.O.A.	Research Ophthalmic Technician	OGCSB, NEI
	Patrick Lopez	C.O.T.	Research Ophthalmic Technician	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Eye Services Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.29	0.21	1.08

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The visual function of patients with ocular diseases or lesions in the visual pathways and of normal subjects is measured using psychophysical techniques. These data are correlated with those obtained with electrophysiological tests of visual function. The results obtained contribute to the diagnosis of ocular and neural disorders that affect vision and are needed to characterize their nature and evolution. They are also valuable in the assessment of how different forms of treatment affect the outcome of these diseases.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The aims of this project are to apply and develop psychophysical techniques for the study of vision in the clinical setting, to characterize the human visual system's normal function, and to analyze the patterns of its alteration in ocular diseases and lesions of the visual pathways.

Methods

Several psychophysical techniques are used: (1) perimetry—visual fields are explored by kinetic quantitative perimetry and statis quantitative perimetry; (2) color vision—central color vision is estimated using HRR pseudoisochromatic plates, Ishihara pseudoisochromatic plates, Farnsworth's Tritan plate, Farnsworth-Minsell D-15 panel, Lanthony's desaturated D-15 panel, Farnsworth-Munsell 100 hue test, and the Nagel anomaloscope; (3) adaptometry— dark-adapted rod and cone thresholds are measured with a modified Goldmann-Weekers adaptometer; (4) spatial vision—the spatial contrast sensitivity function is determined using minusoidal luminance gratings; a two-alternative temporal forced-choice technique is used for a criterion-free judgment of threshold visibility; (5) luminance and chromatic increment thresholds are measured with a two-channel Maxwellian view instrument. This instrument also is used to assess retinal receptor orientation by measuring the Stiles-Crawford effect (SCE of the first kind).

Major Findings

We measured luminance increment thresholds in the visual field of normal subjects to determine whether spatial summation (SS) could be assessed using a standardized perimetric technique. Twenty-five normal volunteers (17 women and eight men, with an age range of 28 to 58 years) were studied. Static thresholds were measured every 2° along the nasal horizontal meridian from 0° (fovea) to 30° in one eye, using a Humphrey Field Analyzer. Five test stimuli (0.009 to 2.334 deg^2 in area, corresponding to Goldmann targets I, II, III, IV, and V) were presented at random. Mean data were modeled well ($r^2 \geq 0.97$) using the following power function: threshold luminance $= C \cdot Area^{-k}$. The value of k was used to quantify the magnitude of SS; k may take values between 0 (no SS) and 1 (complete SS). Threshold luminance was lower when large stimuli were present, giving evidence of SS. Summation was incomplete in all visual field sites tested, and its magnitude increased continuously from fovea to periphery. Values of k were 0.335 at 0°, 0.554 at 10°, 0.655 at 20°, and 0.728 at 30°. Our findings indicate that SS can be measured reliably using stimuli and techniques currently available in automated static perimeters. This method allows the exploration of SS as an estimator of visual function in patients with diseases of the eye and optic pathways.

Significance to Biomedical Research and the Program of the Institute

Psychophysical techniques are noninvasive methods useful in the diagnosis and management of ocular diseases and visual pathway lesions. In addition to the application of validated tests, the development of new techniques contributes to the elucidation of the pathophysical mechanisms of visual disorders.

Proposed Course

Clinical psychophysical studies of visual function in diseases of the eye and visual pathways will be continued. We will introduce modifications that are expected to enhance the diagnostic value of the techniques described.

NEI Research Program

Retinal Diseases—Retinal Neuroscience

Strabismus, Amblyopia, and Visual Processing—Sensory Neuro-Ophthalmological Disorders

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00144-13 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Clinical Electrophysiology of the Visual System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
Others:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
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	Patrick Lopez	C.O.T.	Research Ophthalmic Technician	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Eye Services Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.46	0.46	1.0

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[x] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The visual function of patients with ocular diseases or lesions in the visual pathways and of normal subjects is measured objectively using electrophysiological techniques. These data are correlated with those obtained with psychophysical tests of visual function. The results obtained contribute to the diagnosis of ocular and neural disorders that affect vision and are needed to characterize their nature and evolution. They are also valuable in the assessment of the effects of different forms of treatment on the outcome of these diseases.

PHS 6040 (Rev. 5/92)

Project Description

Clinical Protocol Numbers

91-EI-0026
82-EI-0055

Objectives

The aims of this project are to apply and develop eletrophysiological techniques for the study of visual function in the clinical setting, to characterize the normal electrical activity of the human visual system, and to analyze the patterns of the visual system's alteration in ocular diseases and lesions of the visual pathways.

Methods

The electrophysiological techniques used involve recording potentials (electroculogram) generated by the retinal pigment epithelium, in the neural retina (electroretinogram [ERG]), and the central visual pathways (visually evoked potential [VEPs]). These potentials are elicited by unstructured stimuli (Ganzfeld full-field or focal stimulation) and spatially structured stimuli (sinusoidal gratings or checkerboard patterns).

Major Findings

We studied the effect of red adapting light on the rod-mediated ERG. This study was prompted by the requirements of the standard of clinical ERG, which indicates that recording electrodes should be inserted under "dim red light." Rod-mediated ERGs were recorded in eight normal subjects (five men and three women, ranging in age from 27 to 52 years). After dark adaptation, the recording electrode was inserted in complete darkness infrared. Responses were elicited by a Ganzfeld stimulus of $6.04.10^{-3}$ cd.s/m^2 and were recorded first in total darkness and then under gradually increased red illumination (Wratten 26 filter; $6.23.10^{-4}$ to 14.83.10 cd/m^2 in 12 steps). Waveform, amplitude, and implicit time of the rod response were altered by dim red backgrounds. In five subjects, the rod-mediated b-wave recorded in total darkness showed two peaks whose mean implicit times were 94.73 milliseconds (msec) and 110.12 msec. There were no significant changes in mean b-wave amplitude until background illumination reached $2.14.10^{-2}$ cd/m^2. Four subjects showed a paradoxical increase in amplitude with dim backgrounds (greater than $2.14.10^{-2}$ cd/m^2), thus increasing amplitude variance in this range. With brighter backgrounds b-wave amplitude decreased progressively. B-wave implicit time decreased consistently with background illumination of $2.70.10^{-3}$ cd/m^2 or greater. Our results indicate that the rod-mediated b-wave is affected by dim red illumination. This effect should be considered when analyzing normal and pathological ERGs.

Significance to Biomedical Research and the Program of the Institute

Electrophysiological techniques are noninvasive methods useful in the diagnosis and management of ocular diseases and visual pathway lesions. In addition to validated tests, the new techniques developed contribute to the elucidation of the pathophysiological mechanisms of visual disorders.

Proposed Course

We will continue clinical electrophysiological studies of visual function in diseases of the eye and visual pathways, introducing modifications expected to enhance the diagnostic value of the techniques described.

NEI Research Program

Retinal Diseases—Retinal Neuroscience

Strabismus, Amblyopia, and Visual Processing—Sensory Neuro-Ophthalmological Disorders

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00257-06 OGCSB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visual Function Diagnosis Service

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
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COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Eye Services Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.40	0.15	7.25

CHECK APPROPRIATE BOX(ES)

[x] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither
[ ] (a1) Minors
[ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This general service project provides diagnostic support for all research protocols conducted by the Clinical Sections of the NEI and other Institutes that require an assessment of visual function. Psychophysical and electrophysiological techniques are used to detect and quantify visual loss due to disorders of the ocular media, uvea, retina, optic nerve, and central visual pathways.

Project Description

Objectives

The aim of this project is to provide accurate measurements of visual function for the differential diagnosis of visual loss. The first step in this process is the detection of a visual deficit (*i.e.*, determining whether visual loss is present). The second step is the quantification of a detected deficit. The third step is an analysis of the characteristics of the visual deficit to determine the site of the lesion responsible for this symptom (topographic diagnosis). The final step is correlation with other clinical findings to ascribe the visual deficit to a given pathological process.

Methods

The psychophysical techniques used include commercially available and laboratory-developed techniques for the measurement of visual acuity, visual fields, color vision, dark adaptation, spatial contrast sensitivity, and glare disability.

The electrophysiological techniques used include recording potentials generated by the retinal pigment epithelium (electrooculogram), the neural retina (electroretinogram), and the central visual pathways (visually evoked potentials).

Major Findings

During October 1, 1993, through September 30, 1994, we performed the following tests:

Kinetic perimetry
Static perimetry
Screening perimetry
Manifest refraction
Color vision tests
Adaptometry
Contrast sensitivity tests
Glare disability tests
Ganzfeld electroretinography
Focal electroretinography
Electrooculography
Visually evoked potentials

This represents an increase of 57 percent over the tests performed during the same period of fiscal year 1994.

Significance to Biomedical Research and the Program of the Institute

This project provides all tests of visual function for patients who visit the NEI Eye Clinic. In the majority of ophthalmologic diseases, visual loss is the most meaningful finding. In most clinical research protocols involving diseases of the eye and visual pathways, visual deficit is used as an indicator of the progress of a disease or the effect of a treatment. Therefore, sensitive and accurate measurements of visual function are essential for these clinical research projects.

Proposed Course

The provision of clinical electrophysiological and psychophysical tests of visual function for patients with diseases of the eye and visual pathways will be continued. We will introduce modifications that are expected to enhance the diagnostic value of the techniques described.

NEI Research Program

Retinal Diseases—Retinal Neuroscience

Strabismus, Amblyopia, and Visual Processing—Sensory Neuro-Ophthalmological Disorders

Publications

The nature of this project is such that the results obtained are included in publications listed under other projects.

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